Universidade de Aveiro Departamento de Biologia
Ano 2013/2014

Marta Daniela Lima Braga Jesus Pereira

Directed evolution of lichenicidin

Evolução dirigida da lichenicidina

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro e da Doutora Tânia Isabel Sousa Caetano, Bolseira de Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro

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Dedico este trabalho aos meus queridos pais Maria Manuela e Ernesto assim como, ao meu irmão João.

o júri

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Coli 4Lan

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Lichenicidina, lantipeptidos, lantibiótico, evolução dirigida, mutagenese aleatória, bioengenharia de lantipeptidos.

resumo

A lichenicidina é um lantipéptido da classe II constituído por dois péptidos, Bliα e Bliβ, produzido por diversas estirpes de *Bacillus licheniformis.* Estes péptidos atuam sinergisticamente, de forma a inibirem o crescimento de outras bactérias de Gram positivo, sendo por isso também designados de lantibióticos. Ao contrário de outros compostos antimicrobianos produzidos por bactérias, os lantibióticos são produzidos na sua forma imatura diretamente pelo ribossoma e sofrem diversas alterações pós-traducionais, que os tornam péptidos ativos. Desta forma, torna-se mais fácil a aplicação de técnicas de bioengenharia de modo a obter novas variantes com bioatividade alterada.

Os objetivos deste trabalho foram i) a construção de um sistema de expressão heteróloga da lichenicidina em *E. coli* que permitisse a seleção azul/branco, ii) a construção de uma biblioteca por mutagénese aleatória para cada um dos péptidos que constituem a lichenicidina e iii) a seleção e análise de clones com bioatividade reduzida relativamente ao controlo.

Para a criação do sistema de expressão heteróloga, foram realizados vários ensaios preliminares, que levaram à seleção da estirpe *E. coli* Mach1 como recetora assim como à seleção da temperatura de produção de 37 ºC. Assim sendo, foram construídas duas bibliotecas por mutagénese aleatória dos genes estruturais *licA1* e *licA2*, que codificam os péptidos percursores de Bliα e Bliβ, respetivamente. Subsequentemente, a bioatividade de cerca de 4000 clones de cada biblioteca foi analisada através de bioensaio em agar. Através desta análise, não foi possível detetar nenhum clone com bioatividade aumentada em ambas as bibliotecas. Relativamente à biblioteca do péptido Bliα, cerca de 4,5 % dos clones apresentaram bioatividade nula, enquanto que 2,6 % e 2,2 % dos clones apresentaram atividade muito reduzida e atividade reduzida a metade quando comparada com o controlo, respetivamente. Na biblioteca do péptido Bliβ, os valores foram bastante semelhantes, com cerca de 5,1 % dos clones a exibir atividade nula, 1,4 % com atividade muito reduzida e 3,8 % com atividade reduzida a metade quando comparada com o controlo. A sequência dos genes estruturais de alguns destes clones foram analisados, com o objetivo de identificar as alterações responsáveis por estes fenótipos. Esta análise confirmou a importância dos resíduos conservados para a bioatividade dos dois péptidos. Foi também possível reconhecer que substituições envolvendo aminoácidos carregados e prolinas resultam na produção de péptidos inativos.

keywords

Lichenicidin, lantibiotics, lanthipheptides, directed evolution,random mutagenisis,bioengineering of lanthipeptides

abstract

Lichenicidin is a class II lanthipeptide composed by two peptides, Bliα and Bliβ, that is produced by several strains of *Bacillus licheniformis.* These peptides act synergistically in order to inhibit the growth of other Gram positive bacteria. Due to their antibacterial activity, they are also designated lantibiotics. Unlike other antimicrobial peptides produced by bacteria, lantibiotics are ribosomally synthesized as an immature peptide and suffer several post-translational modifications to achieve their active form. Thus, it is easier the application of bioengineering strategies aiming the creation of new variants with different bioactivity.

The aims of this study were: i) the development a heterologous expression system for the production of lichenicidin allowing a white/blue selection and using *E. coli*, ii) the generation of two random mutagenesis libraries for each one of the lichenicidin peptides and iii) the selection and analysis of clones with reduced bioactivity when compared with the control.

To develop the heterologous system, preliminary tests were conducted that allowed the selection of *E. coli* Mach1 as the recipient strain. In addition, 37 ºC was established as the best temperature for lichenicidin production. Thus, two random mutagenesis libraries of the structural genes *licA1* and *licA2*, encoding the precursor peptides of Bliα and Bliβ, respectively. Subsequently, the bioactivity of around 4000 clones of each library was analyzed by colony-agar bioassay. In the present study, colonies with improved activity were not possible to detect in any of the libraries. Regarding the Bliα library, about 4,5 % of the clones showed null bioactivity, while 2,6 % and 2,2 % of the clones showed very reduced and activity reduced to half when compared with the control, respectively. In the Bliβ library, the values were very similar since about 5,1 % of the clones showed no bioactivity, 1,4 % showed very reduced activity and 3,8 % had activity reduced to half when compared with the control. Some of these clones were selected for sequencing reaction in order to identify the mutations causing the observed phenotypes. This analysis confirmed the importance of the conserved residues for the bioactivity of both peptides. Moreover, it was found that substitutions involving charged amino acids and prolines result in the production of inactive peptides.

Table of contents

List of figures

List of tables

List of abbreviations

Amp	Ampicilin
Cm	Chloramphenicol
Dha	2,3-didehydroalanine
Dhb	Z-2,3-didehydrobutyrine
DNA	Deoxyribonucleic acid
IM	Integral membrane domains
LA	Luria-Bertani Agar
Lan	Lanthionine
LB	Luria-Bertani broth
MeLan	3-metyllanthionine
MRSA	Methicillin resistant *Staphylococcus aureus*
MW	Molecular weight
NRPS	Nonribosomal peptides synthetases
Obu	2-oxobutyryl
OD	Optical density
PCR	Polymerase chain reaction
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultra-Violet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside

List of abbreviations of amino acids

Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartate
Cys	C	Cysteine
Glu	E	Glutamate
Gln	Q	Glutamine
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

1. Introduction

In the microorganisms, there are innumerous sets of microbial defense systems, such as antibiotics, lytic agents and exotoxins. Among the antimicrobial compounds produced by microorganisms are the peptides. These peptides are synthesized by two different mechanisms: ribosomal synthesis and nonribosomal synthesis. Nonribosomally synthesized peptides use large multienzyme complexes designated nonribosomal peptide synthetases (NRPSs), which produce the final peptides. Within this group are the peptides usually referred as the classic antibiotics. Peptides with antimicrobial activity produced by ribosomal synthesis are normally called bacteriocins (Cotter *et al.,* 2005a; Nes *et al.,* 2007a; Arnison *et al.*, 2013).

1.1 Bacteriocins

Bacteriocins can be defined as small and heat-stable bacterially produced antimicrobial peptides that are active against other bacteria. They can have a narrow spectrum (to the same species) or a broad spectrum of activity (across a genera) (Cotter *et al.,* 2005a; Nes *et al.* 2007a). These toxins have been found in a widespread distribution in Bacteria and according to Klaenhammer (1988), 99 % of all bacteria should have the ability to synthesize at least one bacteriocin. Also, these compounds have been described in some lineages of the Archaea. Bacteriocins can be used as preservatives in food industry and as antibiotics in the pharmaceutical industry (Riley, 1998; Riley & Wertz, 2002).

These antimicrobial peptides can be produced by either Gram-positive or Gram-negative bacteria. Bacteriocins produced by Gram-positive bacteria are generally small, consisting of 30-70 amino acids. Gram-positive bacteriocins are normally non-toxic to eukaryotic cells, and they have much broader inhibitory spectra compared to bacteriocins from the Gram-negative bacteria. Gram-negative bacteria generally produce relatively large and heat-labile bacteriocins (except microcins). Colicins produced by *Escherichia coli* are the most extensively studied, and it's now known that its production is generally mediated by the SOS regulon and it is, therefore, mainly produced under stress

conditions, resulting on death of the producing cell and any neighboring cells (Riley & Wertz, 2002; Nes *et al.,* 2007b).

Due to its heterogeneity, the classification of bacteriocins is complex. According to the increasing knowledge of their biosynthesis, structure and mode of action, they have been grouped into four different classes with some subclasses. Different criteria have been used to classify them, such as the producer organisms, molecular sizes, physical properties, chemical structures and mode of action (**Table 1**) (Nes *et al.*, 2007a; Rea *et al.,* 2011).

Due to the limited scope of this thesis we will focus only on lanthipeptides with antimicrobial activity, which are included in Class I bacteriocins.

Table 1: Classifications scheme for bacteriocins. Adapted from Cotter *et al*. (2005a) and Rea *et al*. (2011).

Classification	Description	Remarks
Class I Lanthipeptides with antimicrobial activity- lantibiotics	Small membrane-active peptides (<5 kDa). Contain the unusual amino acids lanthionine, β-methyl lanthionine and dehydrated residues.	Nisin is the most characterized of the Class I bacteriocins.
Class II Non-lanthionine-containing bacteriocins	Unmodified peptides. Small (<10 kDa) heat-stable membrane-active peptides.	Divided into four subgroups: IIa, pediocin-like; IIb, two-peptide; IIc, cyclic; IId, non-pediocin unmodified peptides.
Class III Bacteriolysins	Large (>30 kDa) heat-labile proteins.	Large heat-labile proteins, often with enzymatic activity.
Class IV Complex proteins	Contain lipid or carbohydrate moieties.	–

1.2. Overview of Class I Bacteriocins – the Lanthipeptides

The term lanthipeptide refers to the abbreviation of lanthionine-containing peptides. This term describes all the peptides that undergo the lanthionine- and melthyllanthionine modifications. Lanthipeptides with antimicrobial activity are called lantibiotics (Arnison *et al.*, 2013).

These peptides are small compounds (19-38 amino acids) that are ribosomally synthesized and suffer posttranslational modifications to achieve their biological active forms. All lanthipeptides possess the unusual amino acid lanthionine (Lan) in their structure that is formed by two alanine residues cross-linked via a thioether linkage connecting their β-carbons. In addition, lanthipeptides can also be characterized by the presence of 3-methyllanthionine (MeLan) amino acids. Both (Lan and MeLan) are the unifying structural motif present in all lanthipeptides (Chatterjee *et al.*, 2005; Willey & van der Donk, 2007; Field *et al.*, 2010; Arnison *et al.*, 2013). Commonly, lanthipeptides contain the dehydrated amino acids 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), whereas further modified residues such as lysinoalanine, S-aminOVinyl-D-CySteine, S-aminovinyl-D-methylcysteine and erythro-3-hydroxyaspartic acid are only found in individual peptides (Sahl *et al.*, 2008; Arnison *et al.*, 2013).

The first isolated and arguably the most studied lantibiotic, nisin, is produced by *Lactococcus lactis*. This lantibiotic was discovered in 1928. However its structure was only revealed some years later (in 1971). Based on this discovery, it was proposed that the dehydrated amino acids in lantibiotics were the result of the dehydration of Thr and Ser residues, resulting in Dhb and Dha respectively. It was also suggested that Lan and MeLan rings were made by the reaction of Cys residues with these two dehydrated amino acids (Dha and Dhb) (**Figure 1**). However, this hypothesis was only confirmed after the elucidation of the nucleotide sequence of respective gene clusters (Chatterjee *et al.*, 2005; Willey & van der Donk, 2007; Bierbaum & Sahl, 2009).

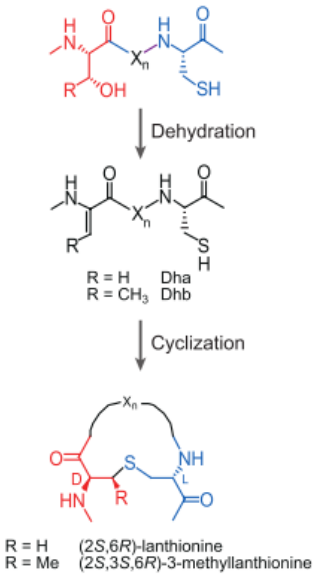

Figure 1: Mechanism of production of lanthionine (Lan) or methyllanthionine (MeLan) residues (Willey and Donk, 2007).

Nowadays there are two classification schemes for lanthipeptides: i) the classification based on the their chemical structure and ii) the classification according to the enzymes that synthesize the Lan and MeLan residues (Chatterjee *et al*., 2005; Nes *et al.,* 2007a; Bierbaum & Sahl, 2009). Considering the second classification scheme, the lanthipeptides are divided in four classes (**Figure 2**) (Knerr & van der Donk, 2012). In these classes, we can find differences in the leader peptide sequence, biosynthetic operon structure, peptide function, and the structure of the mature lantibiotic. Considering the biological activity, only lanthipeptides belonging to class I and class II show antimicrobial activity. The advantages of this classification scheme rely on its simplicity and flexibility to accommodate lanthionine-containing peptides that have not yet been discovered (Willey & van der Donk, 2007).

The lanthipeptides are biosynthesized by the ribosome as a precursor peptide generally designated by LanA. This precursor can be physically divided in a leader and a core peptide. The mature and active lanthipeptides are produced after the intervention of one or more enzymes that install the thioether cross-links in the core peptide, followed by removal of the leader peptide and export to the extracellular space.

In class I lanthipeptides, LanA are modified by two distinct enzymes, LanB enzyme, which dehydrates Thr and Ser residues, and LanC, which mediates the cyclization step. Normally these lantibiotics are more linear than class II lanthipeptides. Class II lanthipeptides are modified by the LanM, which are large (900–1000 amino acids) proteins that exhibit both dehydratase and cyclase activities. The class III lanthipeptides are modified by a trifunctional synthetase bearing an N- terminal lyase domain, a central kinase domain, and a putative C-terminal cyclase domain, which lacks many of the conserved active-site residues found in LanC/LanM. In the more recently described class, class IV, the lanthipeptides are modified by LanL (continuing the common nomenclature for lantibiotic biosynthetic genes), that contains a N-terminal lyase and kinase domains as in class III, but its C-terminal cyclase domains is analogous to LanC (**Figure 2**) (Willey & van der Donk, 2007; Goto *et al.*, 2010; Knerr & van der Donk, 2012).

Lichenicidin is a class II lanthipeptide and is the object of the present work (Begley *et al.*, 2009).

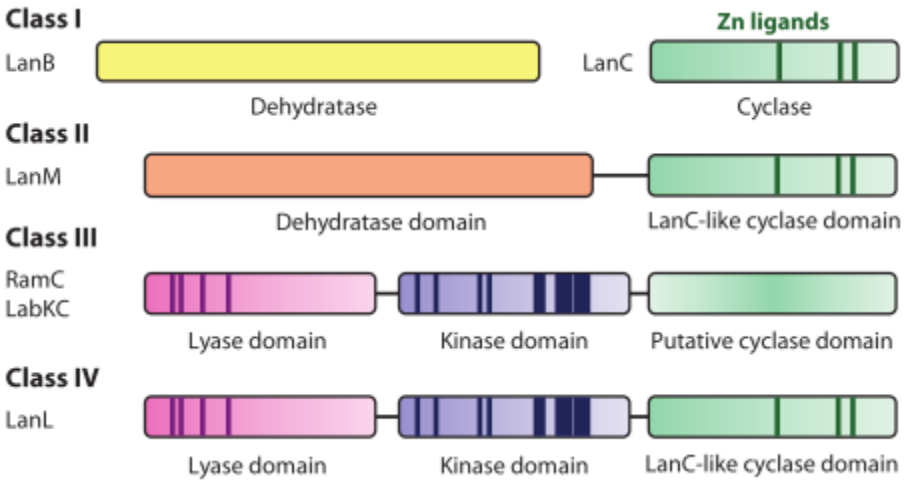

Figure 2: Representation of conserved motifs of the four classes of lanthionine-generating enzymes. Abbreviations: LabKC, labionin synthetase; LanB, lantipeptide dehydratase; LanC, lantipeptide cyclase; LanL, class IV lantipeptide synthetase; LanM, class II lantipeptide synthetase; RamC, SapB-modifying synthetase (Knerr and van der Donk, 2012).

1.2.1 Biosynthesis of Class II lanthipeptides

The biosynthetic gene clusters of lanthipeptides are generally found in the host chromosome (e.g., lichenicidin) or in plasmids (e.g. lacticin 3147) (Chatterjee *et al.*, 2005;

Willey & van der Donk, 2007). The genes for lanthipeptides biosynthesis have been designated with the generic locus symbol *lan* with a more specific designation for each lanthipeptide member (e.g., Lct for lacticin 481, Mrs for mersacidin). Many of these *lan* genes have been sequenced and a high degree of similarity is found on the gene organization of different lantibiotics (Chatterjee *et al.*, 2005).

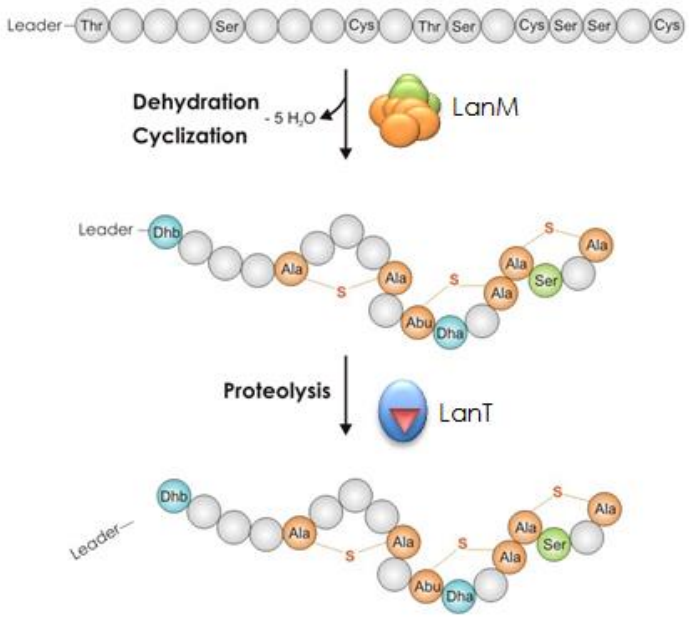

Figure 3: General overview of biosynthesis of class II lanthipeptides (Caetano, 2011).

The gene clusters always comprise structural gene *lanA*, and also the genes encoding the modification enzymes and transporter/protease. The genes can differ in order, complexity and transcriptional organization. Generally, lanthipeptides with other unusual posttranslational modifications, also possess the genes encoding the enzymes required for that process in the same cluster (Chatterjee *et al.*, 2005; Willey & van der Donk, 2007).

Comparison of the gene sequences of different clusters allowed the identification of three genes that are involved in the biosynthesis of all class II lanthipeptides, which are *lanA*, *lanM* and *lanT* (Figure 4). The *lanA* is translated into the LanA precursor peptide, which is biologically inactive because it still contains the N-terminal leader peptide. The leader peptides of class II are rich in aspartate and glutamate residues and finish with a double-Gly motif (includes Gly-Gly, Gly-Ala and Gly-Ser sequences) (Chatterjee *et al.*, 2005). The C-terminal region of the precursor peptide is designated as core peptide. The

Ser and Thr residues can be present on both leader sequence and core peptide, whereas Cys residues are only found in the core peptide segment (Sahl & Bierbaum, 1998; Mcauli *et al.*, 2001; Chatterjee *et al.*, 2005). This is the region where the dehydration and cyclization post-translational modifications are introduced by the LanM enzyme. It corresponds to the mature and active lanthipeptide, after the removal of the leader peptide by the N-terminal protease domain of LanT. This proteolytic processing takes place at the double-Gly motif and can occur before or during export. The ABC transporter encoded by the C-terminal of LanT performs this export. The gene clusters can also contain a second transport system composed by *lanE*, *lanF* and *lanG* genes, which is related with the self-immunity of the producing strain. In some cases, a *lanI* gene is also present, which is believed to be involved also in self-protection (Figure 4). Finally, some clusters also possess genes encoding proteins involved in the regulation of lantibiotic production (LanK and LanR) comprising a two-component sensory system (Figure 4) (Chatterjee *et al.*, 2005; Willey & van der Donk, 2007). The isolation of precursor peptides from the cytoplasm of producing strains has proved quite difficult, suggesting that these have a short half-life, being dehydrated immediately after synthesis (Mcauli *et al.*, 2001).

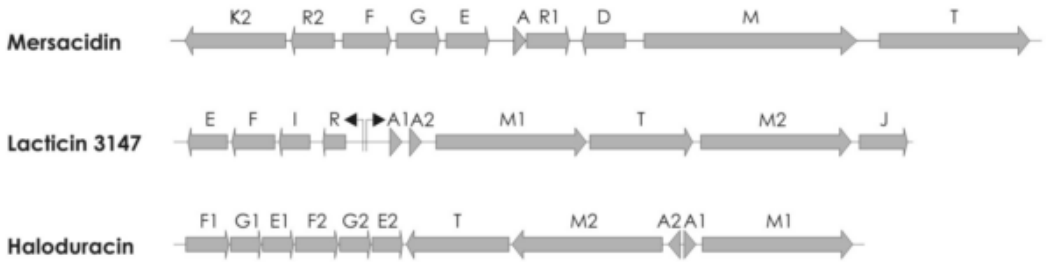

Figure 4: Representation of biosynthetic gene clusters involved in the production of the most representative lanthipeptides from class II (Caetano, 2011).

A growing subgroup within the class II lanthipeptides is the two-component peptides. In this subgroup, two peptides are needed to achieve the potent antimicrobial activity that results from their synergistic interaction. Each peptide is encoded by its own structural gene but are modified by separate LanM enzymes. Generally, a single LanT protein removes the leader peptide and secretes both products. Some of these compounds might undergo an additional N-terminal proteolytic step presumably by the

action of an extracellular protease (Willey & van der Donk, 2007; Knerr & van der Donk, 2012).

1.2.1.1 Lichenicidin – Class II lantibiotic

The lanthipeptides with antibacterial activity are designated as lantibiotics. Thus, lichenicidin is a two-component lantibiotic produced by several *Bacillus licheniformis* strains (Dischinger *et al.*, 2009; Shenkarev *et al.*, 2010; Caetano *et al.*, 2011a). Although not recognized at the time, lichenicidin was firstly detected as a secondary metabolite of *B. licheniformis* I89, which was isolated from an hot spring environment (Mendo *et al.*, 2004). However, the first description of lichenicidin as a two-component lantibiotic was in 2009, involving its production by *B. licheniformis* DSM13 (ATCC 14580). Later, it was also detected in *B. licheniformis* VK21 supernatants (Begley *et al.*, 2009; Dischinger *et al.*, 2009; Shenkarev *et al.*, 2010).

The gene cluster of lichenicidin (**Figure 5**) is located at the far end of the chromosome, and is composed by 14 ORFs. It comprises the two structural genes (*licA1* for the mature α-peptide, Bliα and *licA2* for mature β-peptide, Bliβ), the two bifunctional dehhydratases-cyclases modification enzymes (*licM1* for modification of LicA1 and *licM2* for modification of LicA2), a processing transporter (LicT) that plays an essential role in removal of the leader peptide and the export of Bliα and Bliβ, and a peptidase (LicP) that was involved only in the Bliβ maturation. It was includes the *licX* gene which encodes a small uncharacterized hypothetical protein, but is known that LicX does not play an essential role in lichenicidin production. The role of LicR and LicY in the biosynthesis was not established yet. Finally, the gene cluster contains a group of 5 ORFs, putatively involved in lichenicidin immunity (Begley *et al.*, 2009; Dischinger *et al.*, 2009; Caetano *et al.*, 2011b).

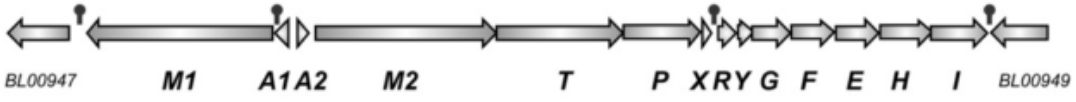

Figure 5: Representation of biosynthetic gene clusters involved in the production of lichenicidin (Caetano *et al.* 2011a).

Both unmodified forms of lichenicidin, encoded by *licA1* and *licA2*, possess a leader peptide with 42 and 34 amino acids respectively (Begley *et al.*, 2009). A double-Gly motif was identified as the proteolytic site for LicT. In lichenicidin propeptide sequence a high number of Ser and Thr especially at the N-termini is observed (Begley *et al.*, 2009; Caetano *et al.*, 2011a).

Our group developed the first heterologous expression system for a class II two-component lantibiotic gene cluster in *E. coli.* This system was developed for the lichenicidin produced by *B. licheniformis* I89. This allowed the production of new lichenicidin variants and permitted the identification of essential residues for biosynthesis and antibacterial activity. In addition, it enabled to confirm the structure of the peptides, previously proposed (Caetano *et al.*, 2011a). At the structural level (Figure 6), the first amino acid of both Bliα and Bliβ undergoes dehydration, followed by a deamination resulting in a 2-oxobutyryl (Obu1) residue. The same was also described for other lantibiotics such as Pep5 and Lactβ peptides (Caetano *et al.*, 2011a). A study performed by Caetano et al. (2011a) described an Ala-scan for all positions containing Ser and Thr residues, which confirmed that Ser30 escapes dehydration in Bliα and also Thr10, Ser21 and Ser30 remain unmodified in the Bliβ. Also, it was found that in Bliα, Ser5 with Cys7 are the amino acids involved in the Bliα A-ring formation and in Bliβ, Ser7 is the residue involved in Lan formation with Cys11. Moreover, the pattern of lanthionine rings (especially B, C, and D rings) seems to be conserved among two-component lantibiotics, indicating their importance at the biological activity level. Mersacidin has a Glu17 residue that is essential for binding to the lipid II target. In lichenicidin Bliα, the homologous Glu26 in also important for bioactivity.

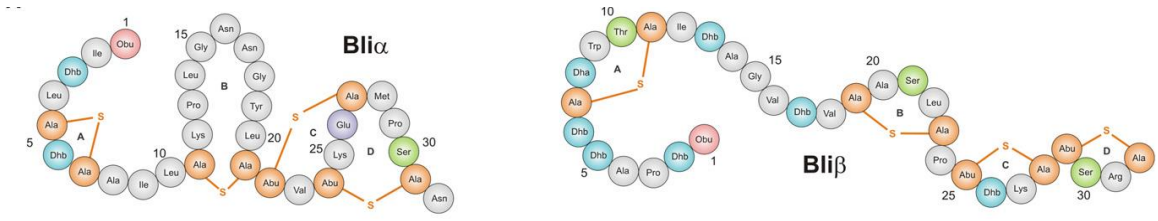

Figure 6: Proposed structures of Bliα and Bliβ peptides of the lichenicidin complex (Caetano *et al.* 2011a).

1.2.2 Mechanism of action of lantibiotics

Lantibiotics have different mechanisms of action. While, some bind to lipid II and inhibit the cell wall biosynthesis, others attack the membrane of bacteria forming pores. Some compounds have a double mode of action that combines inhibition of peptidoglycan biosynthesis by binding and dislocation of lipid II with pore formation in bacterial membranes. All functions can be combined in a single molecule (e.g. Nisin) or can be implemented in a combination of two functionally specialized peptides (e.g. Lacticin 3147). In two-peptide lantibiotics, it is believed that the mechanism of action involves the interaction of both peptides. It is thought that α-peptide binds to lipid II on the wall and then forms a complex with the elongated β-peptide. Subsequently, the β-peptide forms an ion-conducing pore in the membrane of the target cell, causing a rapid efflux of K^+ and phosphate ions inducing an immediate dissipation of the membrane potential and hydrolysis of intracellular ATP, leading to cell death. This system can be found in lacticin 3147 lantibiotic (Wiedemann *et al.*, 2006; Dischinger *et al.*, 2009; Shenkarev *et al.*, 2010).

It is well known that the lichenicidin complex is active against methicillin-resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes, Micrococcus luteus* ATCC 9341*, Bacillus subtilis, Enterococcus faecium, Enterococcus faecalis and Haemophilus influenzae* (Mendo *et al.*, 2004; Begley *et al.*, 2009; Dischinger *et al.*, 2009; Caetano, 2011). It is believed that the mechanism of action of lichenicidin should be very similar to that previously described for other two-peptide lantibiotics, especially haloduracin and lacticin 3147. Thus, the α-peptide binds to lipid II and subsequently the β-peptide involved in this complex in a 1:1 stoichiometry is likely to interact with the membrane forming pores (Shenkarev *et al.*, 2010).

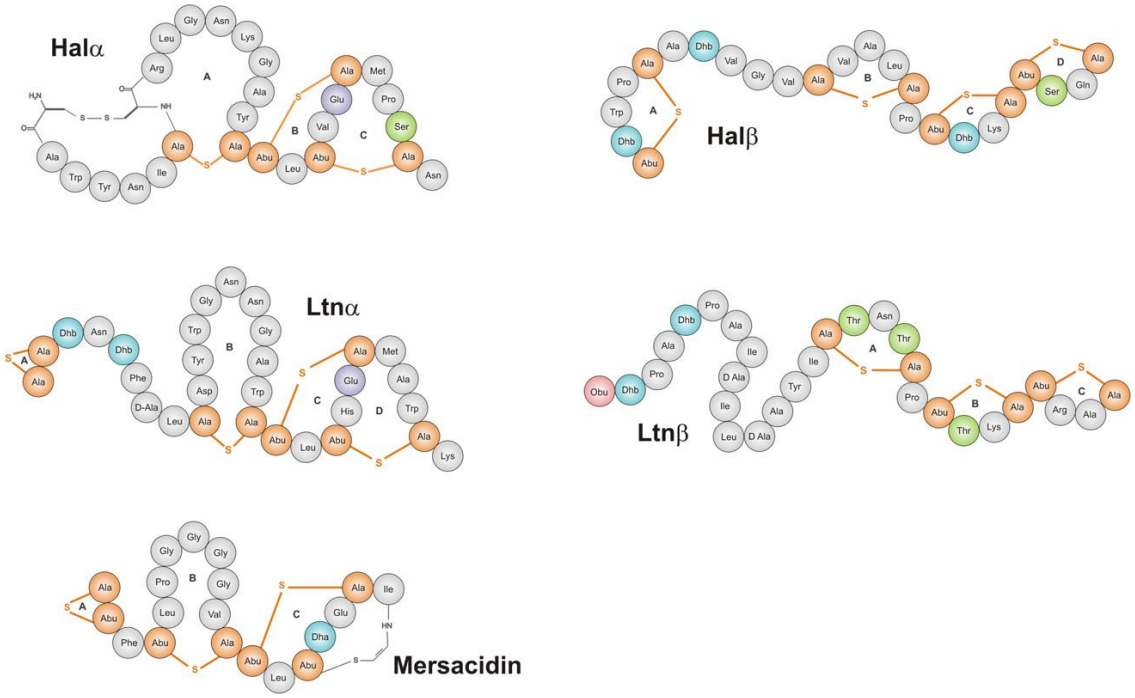

Figure 7: Proposed structures of Mersacidin, halodurain (Halα and Halβ) and lacticin 3147 (Ltnα and Ltnβ) (Caetano *et al.* 2011a).

1.2.3 Self-protection of the producer strain

As discussed before, lantibiotics are potent antimicrobials, with a broad range of target strains. These substances need to be synthesized and exported from the producer strain to the extracellular space. Therefore, the producer strain must defend itself from its own product. Consequently, it must have mechanisms of self-protection preventing cell death caused by its own bacteriocin, a process called immunity.

The producer strain can adopt two types of mechanism: i) individual immunity proteins (normally called LanI proteins), or ii) an ABC transporter (usually composed by two or three subunits called LanFE(G)). It is also possible that both mechanisms can act in combination (e.g Nisin). A third member of the immunity system, LanH can also be present. This protein was thought to act as an ancillary protein for the assembly of a functioning ABC transporter (**Figure 8**) (Draper *et al.*, 2008; Dischinger *et al.*, 2009). The ABC transport systems are one of the most abundant families of proteins in nature. They

are constituted by an ATPase domain of bind and perform the hydrolysis of ATP to ADP. The proteins of the system are composed by two hydrophobic integral membrane domains (IM) where the ligation of the substrate occurs and two hydrophilic cytoplasmic domains carrying the ABC system where the ATP binds and is hydrolysed, proceeding to the import and export of the molecules. The LanI peptide can be found attached to the membrane or free in the outside of the cell. LanI can also function as a single peptide or can form a homodimer. Usually both forms bind to the lantibiotic and forms aggregates. In the NisI case, it is believe that NisI binds and aggregates with nisin preventing the formation of pores (Draper *et al.*, 2008).

Regarding the two-component lantibiotics, there is little information about their immunity systems. In this type of peptides it is not apparent whether they protect cell against one particular peptide or both. Instead, it is thought that they prevent the interaction of the two peptides, thus reducing their activity and potency (Draper *et al.*, 2008). Regarding lichenicidin self-protection it is known that the *licFGEHI* immunity genes are not essential for peptide production when it is produced in the heterologous system in *E. coli* (Dischinger *et al.*, 2009; Caetano *et al.*, 2011b).

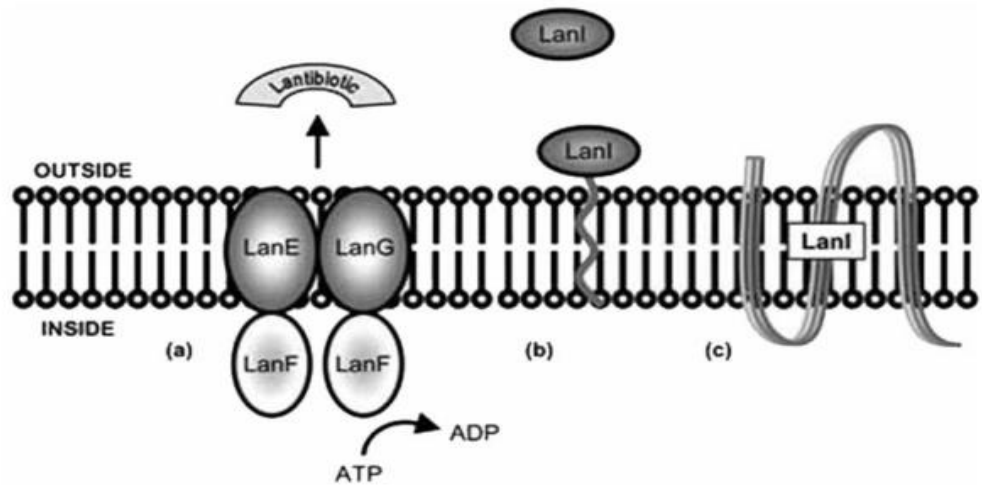

Figure 8: Structural organization of the two type mechanisms of immunity system. LanF functions as an ATPase, while LanE/G form the membrane spanning domain of the ABC transporter (a)**.** The LanI peptide can be found attached to the membrane or found free in the supernatant as observed with NisI (b). LanI peptides can also be integral membrane proteins with three putative transmembrane regions (c) (Draper *et al*. 2008).

1.3 Directed Evolution

Natural evolution produces a large number of proteins and enzyme variants, and these variants exhibit their function with high specificity and efficiency. These changes have adjusted species only to perfect their physiological role and therefore their specificity and stability are usually far away from what biotechnology industry needs (Field *et al.*, 2007, 2010; Labrou, 2010). Directed evolution is an *in vitro* fast process for generation of new variants of proteins, enzymes, metabolic pathways or even entire genomes variants with new and desired properties (Field *et al.*, 2007; Labrou, 2010).

Directed evolution process was a powerful tool especially for proteins whose crystal structures are unavailable or for those which relationships between structure and function continue to be recognized. Thus, a successful directed evolution experiment implies an efficient library construction (that must be as large and diverse as possible) and a robust high-throughput screening of the mutant library (**Figure 9**) (Brakmann & Schwienhorst, 2004; Vanhercke *et al.*, 2005; Wang *et al.*, 2006).

Random mutagenesis is a strategy where random point mutations are introduced into targets (e.g. gene, whole genome). This methodology is usually used to create mutant libraries for protein engineering applications, with the ultimate goal of generating protein variants with desired properties. The selection of the appropriate method for random mutagenesis, takes into account the most important performance criteria which include an impartial mutation spectrum and a controllable mutation frequency. There are a lot of different techniques that can be applied, as enzymatic strategies that explore the properties of DNA modifying enzymes like DNA polymerase, in “error-prone” PCR. Other alternatives are the purely chemical methods and the cell based mutagenesis methods, which are based on the increase of errors during DNA replication (Brakmann & Schwienhorst, 2004; Rasila *et al.*, 2009; Labrou, 2010). However, the most common technique is random PCR mutagenesis due to its simplicity and versatility. This technique has a mutation frequency of around 10 % per nucleotide position. Changing some PCR conditions allows the rate of the mutation to be adjusted to the appropriate level (Brakmann & Schwienhorst, 2004).

Usually, the experimental cycle of directed evolution using random mutagenesis consists in some important steps. At the beginning, a DNA sequence that encodes the desired protein is chosen and this sequence is submitted to a step of mutagenesis, to introduce some random point mutations, followed by the introduction of the mutated DNA in an expression vector that is incorporated into a host cells. Then, a screening to select the transformants that contain the encoded sequence as well as the desired properties is performed. In the end, the selected sequence can be re-amplified and the cycle can be repeated again for further improvement if necessary (**Figure 9**) (Labrou, 2010).

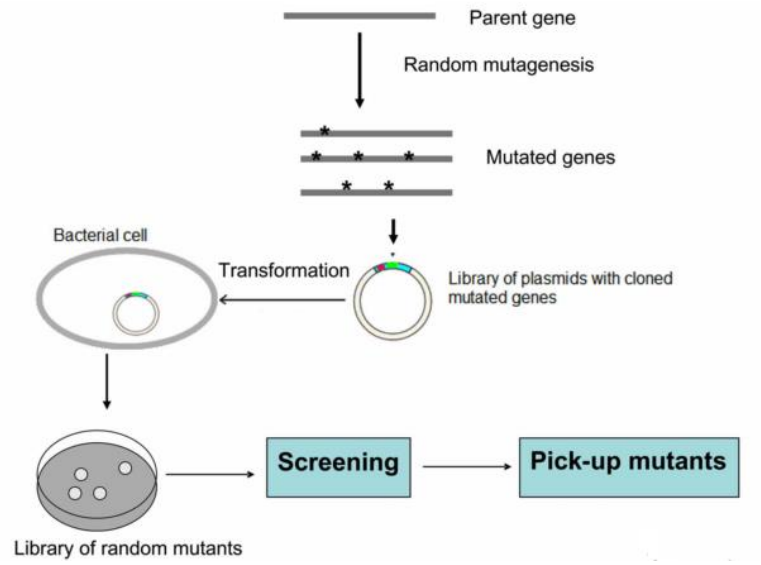

Figure 9: Experimental steps of an in vitro directed evolution process. A parent DNA sequence encoding for a desired protein is chosen. Sequence diversity is created through a random mutagenesis step, (the symbol * represents point mutation). The library of DNA sequences is ligated into an expression vector. Recombinant clones are transformed into host cells, and the protein variants are expressed. A screening or selection procedure is employed next to isolate the transformants (Labrou, 2010).

1.3.1 Random mutagenesis applied to the bioengineering of lanthipeptides

Lanthipeptides have a ribosomal origin that turns it easier the application of strategies to produce modifications on the peptide, enabling the creation of new variants with altered biological, chemical and physical properties. Such modifications can be generated by site-directed mutagenesis or by random mutagenesis (Field *et al.*, 2007,

2010). The site-directed mutagenesis ensures the substitution of a specific single amino acid, however it is time consuming. Thus, random mutagenesis has been an appealing alternative. However, few lantibiotics have been subjected to this process. For instance, for class I lanthipeptides this approach was used only for nisin and for class II was used for lacticin 3147.

A random mutagenesis library was performed to nisin and around of 8000 mutants were screened and only one variant exhibited enhanced efficacy against both Gram-positive and Gram-negative bacteria (Field *et al.*, 2012).

Regarding Lacticin 3147, a two-component lantibiotic like lichenicidin, the random mutagenesis library was performed by mutating simultaneously both structural genes. After the construction of this library, around 1500 mutants were screened to identify mutations with impact on the bioactivity and mutations involved in improved bioactivity were not identified. In addition, it was confirmed the importance of some residues that caused a decrease and abolishment of the bioactivity (Field *et al.*, 2007, 2008).

2. Objectives

Bioengineering of lichenicidin can be important to better understand its biosynthesis and its mechanism of action. Also, it would be important to obtain new variants with improved bioactivity. An attractive technique to generate these variants is the use of random mutagenesis. Thus, the objectives of this work were:

i) Construction of a heterologous expression system of lichenicidin in *E. coli* allowing white and blue selection;
ii) Generation of two independent libraries by random mutagenesis for each of the lichenicidin peptides (Bliα and Bliβ);
iii) Identification of mutations with negative and positive impact on the bioactivity of the peptides produced.

3. Material and Methods

The following flowchart shows the experimental procedures employed for establishment of the white and blue selection system, the generation of mutants to both peptides of lichenicidin and the selection of these mutants (**Figure 10**).

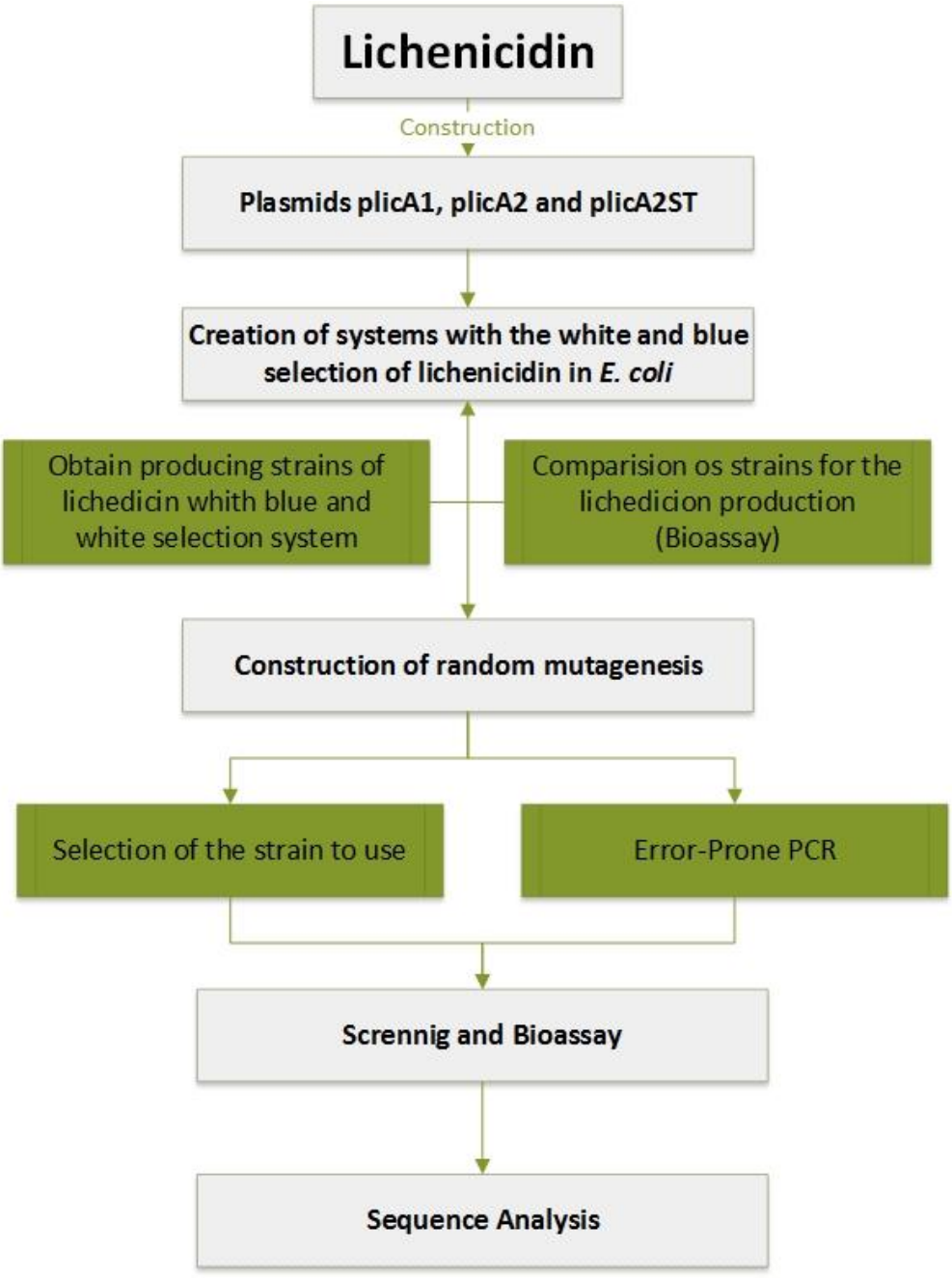

Figure 10: Schematic representation of the workflow of the present work.

3.1 Vectors and strains

In this study, different strains of *E. coli* of which some comprising fosmid and plasmids with parts of the lichenicidin gene cluster were used. The following tables show the strains and vectors used.

Table 2: List of general strains used in this study. ATCC (American Type Culture Collection); MUL (University of Lisbon Microorganims Collection).

Strain	Description/ Genotype	Phenotype	Source
E. coli DH5α	recA1relA1gyrA96deoRnupGΦ80dlacZΔM15 Δ(lacZYAargF)U169, hsdR17(rK- mK+), λ–	-	MUL
E. coli Mach1	F- ϕ80(lacZ)ΔM15 ΔlacX74 hsdR(rK-mK+) ΔrecA1398 endA1 tonA	-	Invitrogen™
E. coli Top10	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG	-	Invitrogen™
E.coli Blic5ΔA1	*E. coli* BL21-Gold(DE3) containing the pLic5ΔA1 fosmid (pLic5 with *licA1* gene deleted)	Cm^R	(Caetano, *et al.*, 2011a)
E.coli Blic5ΔA2	*E. coli* BL21-Gold(DE3) containing the pLic5ΔA2 fosmid (pLic5 with *licA2* gene deleted)	Cm^R	(Caetano *et al.*, 2011a)
M. luteus ATCC 9341	Indicator strain	-	MUL

Table 3: Plasmid and fosmids used in this study, MW refers to the molecular weight of the respective vector.

Vectors	Source	MW	Selective marker	Description
pUC19a	Fermentas	2.7Kb	Amp^R	High copy number *E. coli* plasmid; region of *E. coli lac* operon, promoter Plac, *lac* repressor binding site and the 5'-terminal part of the *lacZ* gene encoding the N-terminal fragment of beta-galactosidase, includes *Nco*I and *Nhe*I restriction sites.
pLic5	This group	34.8Kb	Cm^R	pCC2FOS fosmid containing all the the genes for lichenicidin production.
pLic5ΔA1ΔA2	This group	34.1Kb	Cm^R	pLic5 fosmid with all the genes for lichenicidin production, except the structural genes A1 and A2.
pUClicA1	This group	3Kb	Amp^R	pUC19a plasmid with the *licA1* gene into the MCS.
pUClicA2	This group	3Kb	Amp^R	pUC19a plasmid with the *licA2* gene intro the MCS.
pUClicA2ST	This group	3Kb	Amp^R	pUC19a plasmid with the licA2 gene. Instead of the TAG stop codon it has the TAA stop codon.

3.2 Media and growth conditions

E. coli strains were grown in Luria-Bertani agar (LA) plates at the appropriated temperature. Whenever extraction of DNA was necessary, Luria-Bertani broth (LB) was used for general liquid growth of *E. coli* strains at 37 ^{o}C, and at 180 rpm. For lichenicidin production M medium was used that consisted of: 10 g/L of NaCl, 10 g/L of trytptone, 5 g/L of yeast extract, 10 g/L of KH2PO4, pH was adjusted with NaOH to 6.5 (Mendo *et al.*, 2004). For solid-based production assays M medium was supplemented with 20 g/L of agar prior to autoclaving. *Micrococcus luteus* ATCC 9341 was used as the indicator strain in the bioassay to evaluate lichenicidin production. The strain was maintained in tryptic soy agar (TSA), and liquid cultures were performed in tryptic soy broth (TSB) at 37 ^{o}C and 180 rpm. For routine bioactivity screening, the selection was performed in LA plates containing the following antibiotic concentrations: chloramphenicol (Cm), 12.5 μg ml^{-1} and ampicillin (Amp), 100 μg ml^{-1}.

3.3 Construction of plasmids plicA1, plicA2 and plicA2ST

In this study, three plasmids were used. The first plasmid contained the structural gene for *licA1* (plicA1). A second plasmid contained the gene for the *licA2* production (plicA2). However, when the latter plasmid was used, it was observed that colonies that should have a white color, presented a bluish color that may correspond to false positive clones. One possible justification for this color could result from a deficient stop codon that is less favorable in *E. coli* strains. Thus, it was necessary to construct another plasmid containing the *licA2* gene and where TAG stop codon was replaced by TAA a more efficient stop codon for *E. coli*.

The amplification of *licA2* was performed by NZYProof DNA Polymerase (Nzytech) in a 25 μL reaction containing 0.25 μL of dNTPs mix (0.2 mM), 2.5 μL of Reaction buffer (10X), 0.75 μL of each primer (10 pmol/μL), 0.01-0.5 μg of total DNA of *B. licheniformis* I89 and 0.25 μL of NZYProof DNA polymerase (0.625 U). The primers used are listed in **Table 4**.

The PCR program had an initial denaturation step, 95 ^{o}C for 2 min, followed by a denaturation step 95 ^{o}C for 30 seg, annealing at the specific temperature (**Table 4**) and extension at 72 ^{o}C for a given time (**Table 4**). These 3 last steps were repeated 30 times

followed by a final extension step at 72 ºC for 5 min. PCR product was analyzed by electrophoresis 1 % agarose gel (**Appendix 1**) and later purified by NZYGelpure kit from Nzytech according to the manufacturer's instructions (**Appendix 2**). DNA concentration it was determined using Qubit® (**Appendix3**).

Table 4: List of primers used to amplify *the licA2* and respective sequences, annealing temperatures, expected size of each amplicon, the extension time for each target gene and the respective restriction enzyme.

Primer Name	Primer sequencing (5'→3')	Annealing Temperature (ºC)	Expected amplicon (bp)	Extension Time	Restriction enzyme
Comp_licA2_BamHI_Rv	TATGGATCCCTAGCATCGGCTTGTACAC	59	300	60 seg	*Bam*HI
Comp_licA2_NcoI_Fw	TATCCATGGCTATGAAAACAATGAAAAATTC				*Nco*I

To introduce the amplified *licA2ST* fragment into the vector pUC19a, it was necessary to make a digestion of both elements before ligation. Plasmid digestion was performed in a final volume of 20 µL containing 600 ng of pUC19a DNA. PCR product digestion was carried out in a final volume of 40 µL with 1000 ng of *licA2ST*. In the plasmid reaction, 1 µL of each enzyme (*Nco*I and *Bam*HI) and 2 µL of the 10X FastDigest Buffer (Thermo Scientific) where used. In the reaction of the PCR product, the volumes employed were twice as those used in plasmid reaction. The digestion was performed at 37 ºC for 5 min, and followed by purification using the NZYGelpure kit from Nzytech according to the manufacturer's instructions (**Appendix 2**).

Ligation reaction was performed in a total volume of 20 µL containing 50 ng of pUC19a DNA, 150 ng of *licA2ST* DNA, 1X T4 DNA ligase buffer and 1 µL of T4 DNA ligase (Fermentas). Reaction was incubated at 22 ºC for 30 min on a thermocycler (BioRad) and posteriorly conserved at -20 ºC until further use.

Competent cells were prepared as described in 3.7.1 and transformation was performed according to 3.7.2.

3.4 Development of heterologous expression system of lichenicidin in *E. coli*.

3.4.1 Blue and white selection of lichenicidin producing strains

In all the 3 *E. coli* strains used (*E. coli* Top10, *E.coli* DH5α and *E.coli* Mach1) the fosmid containing all the genes for lichenicidin production, with exception of the structural genes, was introduced (plic5ΔA1ΔA2). The fosmid was extracted by alkaline lysis (**Appendix 4**) and transformed by heat-shock in chemically-competent cells (see 3.7.1 and 3.7.2).

Table 5: List of primers used to amplify *licT* and *licP* and respective sequences, annealing temperatures, expected size of each amplicon and the extension time for each target gene

Primer Name	Primer sequencing (5'→3')	Annealing Temperature (ºC)	Expected amplicon (bp)	Extension Time
Comp_licT FW	AAGGAGATATACATATGTTT TTTCATAAGACACCGTT	52	3600	3 min and 50 seg
Comp_licP RV	GGTGGTGGTGCTCGAGTCA CTCCTTGTTCATCATTTTC			

Fosmid incorporation was confirmed by PCR. The amplification reaction was performed with *Taq* DNA polymerase (Nzytech) as described in **Appendix 1** and using the primers and annealing temperatures indicated in **Table 5**. PCR products were analyzed by electrophoresis 1 % agarose gel (**Appendix 1**).

Subsequently, each strain containing the pLic5ΔA1ΔA2 fosmid (*E. coli* Top10 pLic5ΔA1ΔA2, *E.coli* DH5α pLic5ΔA1ΔA2 and *E.coli* Mach1 pLic5ΔA1ΔA2) was transformed with a plasmid containing one of the structural genes (plicA1, plicA2 and plicA2ST). These plasmids were extracted with the Plasmid DNA Kit from KOMABIOTECH as described in **Appendix 4**. In this step were used the strains *E. coli* Mach1 plicA2ST to extract the plasmid containing the gene encoding *licA2* with the new stop codon (plicA2ST), *E. coli* DH5α plicA1 to extract the plasmid containing the gene encoding *licA1* (plicA1) and *E. coli* DH5α plicA2 to extract the plasmid containing the gene encoding *licA2* (plicA2). The

strains were inoculated in 5 mL of LB medium supplemented with Amp and grown overnight at 37 ^{o}C and 200 rpm.

Table 6 List of primers used to amplify the MCS of pUC19a and respective sequences, annealing temperatures, expected size of each amplicon and the extension time for each target gene

Primer Name	Primer sequencing (5'→3')	Annealing Temperature (^{o}C)	Expected amplicon (bp)	Extension Time
Puc19 FV	GTAAAACGACGGCCAGT	56	300	45 seg
Puc19 TC RV	CTTCCGGCTCGTATGTTG			

After transformation cells were spread on LA plates supplemented with Cm, Amp and X-Gal and were incubated overnight at 37 ^{o}C. A PCR was employed to confirm the presence of the desired plasmid in the transformed cells. Only white clones were selected for colony-PCR. The amplification reaction was performed with *Taq* DNA polymerase (Nzytech) as described in **Appendix 1**, using the annealing temperatures indicated in **Table 6.** PCR products were analyzed by electrophoresis 1 % agarose gel (**Appendix 1**).

In order to confirm if the obtained strains were producing the expected peptides, a colony-bioassay was performed. Tis bioassay was performed, in a Petri dish containing TSA and *M. luteus* (OD_{600} reached 0.02) where each strain was inoculated together with the complementary strain (Bli5ΔA1 or Bli5ΔA2). The aim of this assay is to verify the presence of inhibition halos between the two strains after incubation overnight at 37 ^{o}C.

3.4.2 Comparison of Bliβ and Bliα production by the different strains

In order to select the appropriate strain and temperature to construct and screen the final library, the comparison of production in agar medium was performed.

3.4.2.1 Quantification by colony-bioassay

In this assay, 20 mL of agarized M medium was inoculated with one colony of each strain. This was performed in duplicate and one set was incubated overnight at 37 ^{o}C and the other at 30 ^{o}C. After incubation, the plates were exposed to UV radiation for 15 min and overlaid with 15 mL of TSA containing *M. luteus* at a final OD_{600} of 0.02 and 1.875 µL of the respective supernatant prepared as described below (**3.4.2.2**). The inhibition zones were measured after an incubation period of 16-18 h, at 37 ^{o}C. The same assay was performed with *E. coli* Mach1pLic5, *E. coli* Top10pLic5 and *E. coli* DH5αpLic5 strains as controls. Since these strains have the entire gene cluster for the production of lichenicidin, it was not necessary to add the supernatant in the medium.

3.4.2.2 Preparation of supernatants

These generated strains can only produce one of the peptides, either the Bliα (if they have plicA1) or Bliβ (if they have plicA2 or plicA2ST) and therefore they do not exhibit activity against *M. luteus* unless they are supplemented with the complementary peptide in the agar medium (either beta or alpha respectively) Thus, the peptides were supplied as supernatants: the Bliα as supernatant of the *E. coli* Blic5ΔA2 strain and the Bliβ as supernatant of *E. coli* Blic5ΔA1 strain. To obtain these supernatants, *E. coli* Blic5ΔA1 and *E. coli* Blic5ΔA2 were pre-cultured in LB medium supplemented with 12.5 µg/mL of Cm, at 37 ^{o}C, 180 rpm, overnight. In the following day, 300 µL of this pre-cultured were inoculated into 50 mL of M medium. After 24 h at 37 ^{o}C, 180 rpm, the culture was centrifuged twice at 10 000 rpm for 5 min. The supernatant was collected and filtered through a 0.02 µm nitrocellulose filter and stored at -20 ^{o}C until further use.

3.5. Random mutagenesis library of Bliα and Bliβ peptides

3.5.1. Construction of the library

Random mutagenesis required purified plicA1 and plicA2ST DNA which was performed as described in **Appendix 4**. GeneMorph II Random Mutagenesis kit (Agilent) was used to perform random mutagenesis on the extracted plasmids. The kit contains the Mutazyme II that allows a mutation rate of 1 to 16 mutations per kb. The desired mutation rate can be controlled by varying the initial amount of target DNA in the reaction or by changing the number of amplification cycles. To introduce a maximum of 1.35 base changes on the cloned fragments, a dilution of pDNA was performed to obtain an initial amount of the target gene of 100 ng. The amplification reaction was performed in a 50 µL total volume and the primers used (**Table 7**) were mixed together, in order to obtain a final concentration of 250 ng/µL of each primer. 0.5µL of primer mix was added to the reaction along with, 1 µL of dNTPs mix (25 mM), 2.5 U of Mutazyme II DNA polymerase and 9.09 µL of target DNA was used at a final 20 ng/µL concentration. The amplification initial denaturation step was performed at 95 ^{o}C by 2 min, followed by 30 cycles of denaturation at 95 ^{o}C for 30 sec, annealing at 59 ^{o}C and extension at 72 ^{o}C for 1 min. The final extension step was performed at 72 ^{o}C for 10 min.

The amplificated product was treated with 1 µL of Dpn1 for 1h at 37 ^{o}C. Subsequently, the PCR product was run in an agarose gel and further purified with NZYGelpure kit (Nzytech; **Appendix 2**).DNA concentration was determined using Qubit ® (**Appendix 3**).

Then the PCR product (1000ng) and the plasmid pUC19a (1000ng) were digested with the appropriate enzyme. In both reactions 1 µL of each restriction enzyme was added (BamHI and NcoI) to the reaction together with 1X FastDigest Buffer (Thermo Scientific). The mixture was incubated at 37 ^{o}C for 30 min. Both reactions were subsequently purified using the NZYGelpure kit (Nzytech; **Appendix 2**).

Table 7: Primers used to amplify *licA1 and licA2* for random mutagenesis method and colony-PCR screening. Primer sequence, annealing temperatures, expected size of each amplicon and the extension time for each target gene was are also included.

Primer Name	Primer sequencing (5'→3')	Annealing Temperature (°C)	Expected amplicon (bp)	Extension Time
Puc_mut_Rv 2	GGTACCCGGGGATCCTTA	59	300	1 min
Puc_mut_Fw	GAA ACA GCC ATG GCT ATG			

Ligation reaction was performed in a final volume of 20 µL, containing 16.8 ng of purified and digested PCR product, and 50 ng of pUC19a, 2 µL of 1X T4 DNA ligase buffer and 1 µL of T4 DNA ligase (Fermentas). The reaction was incubated at 22 °C for 2h on a thermocycler (BioRad) and desalted using a membrane MF™ – Membrane filters (Millipore) placed in a surface of a petri dish containing distilled water during 30 min.

Finally, 5 µL of each ligation were mixed with 50 µL of *E. coli* Mach1ΔA1ΔA2 electrocompetent cells (See 3.7.3). The electroporation was then performed using 1 mm gap electroporation cuvettes using the MicroPulser Electroporator (BioRad) (See 3.7.4). 995 µL of LB were added to the cuvettes and after 1 hour at 37 °C, the culture was distributed in 9 tubes containing 100 µL of the culture and 50 µL of glycerol at 45 %. The tubes were then stored at -20 °C until further use. When the culture was necessary, 10 µL of culture together with 90 µL of LB were spread in LB agar plates containing 12.5 µg/mL of Cm, 100 µg/mL of Amp and 100 µg/mL X-Gal.

3.5.2 Screening of the library and bioassay

Approximately 4000 white clones from each library were picked into LA plates supplemented with Amp and Cm. The plates were incubated overnight at 37 °C, in order to further test their antibacterial activity.

The bioactivity of each clone was then evaluated by colony-bioassay. Square plates were prepared with 30 mL of agarized M medium. 11 clones and the respective control (*E. coli* Mach 1ΔA1ΔA2 pLicA1 and *E. coli* Mach 1ΔA1ΔA2 pLicA2ST) were selected and

incubated in each plate overnight at 37 ºC. After incubation, the plates were exposed to UV radiation for 15 min. Finally colonies were overlaid with 15 mL of TSA with *M. luteus* at a final OD_{600} of 0.02 and 3.125 µl of the respective supernatant (see preparation at **3.4.2.2**). The visualization of inhibition areas was possible after overnight growth at 37 ºC.

These selected clones were grouped into 4 distinct groups; null activity (NA), very low activity (VLA), reduced activity (RA) and high activity (HA) always in comparison whit the control. In order to confirm the higher activity of some clones, a dot-colony-bioassay was performed. To make this dot-colony-bioassay, each clone was pre-cultured into 3 mL of LB medium supplemented with 12.5 µg/mL of Cm and 100 µg ml^{-1} of Amp and grown overnight, at 37 ºC, 180 rpm. 30 µL of this pre-cultured was inoculated into 3 mL of M medium and incubated at 37 ºC, 180 rpm, overnight. After 16-18 h, 2 µL of each clone was placed into the plate containing agarized M medium. After overnight incubation at 37 ºC the plates were exposed to UV radiation for 15 min. Finally colonies were overlaid with 15 mL of TSA with *M. luteus* at a final OD_{600} of 0.02 and 3.125 µl of the respective supernatant.

After the confirmation of some bioactivities, around 30 clones of each group were sequenced, following the plasmid extraction (see **Appendix 4**).

3.6 LC-ESI-MS analysis

3.6.1 Extraction and analysis of the peptides

Clones having mutation that do not involve amino acids essential to the bridge formation were selected to a LC-ESI-MS analysis. To obtain this extracts, a pre-inoculum of each clone in 5 mL of medium M, with Cm at 12.5 µg ml^{-1} and Amp at 100 µg ml^{-1} was performed and clones were grown at 37 ºC, 180 rpm, overnight. In the next day, the OD of each culture was measured. If the OD it was similar, it was not adjusted. If not, needs to be adjusted. 300 µL of which culture was used to seed the 30 mL of medium M and grow for 24 hours at 37 ºC. On the following day, the culture was transferred to a falcon and 5 ml of Butanol was added. After 1 hour at 180 rpm, a centrifugation step was

performed at 10 000 rpm by 5 min. 1 ml of the upper phase was collected, placed in a 1,5 tube and a evaporation step at 50 ^{o}C was performed. Tubes were stored at -20 ^{o}C until be analyzed.

The LC-ESI-MS analysis was performed according to Caetano *et al.* (2011a).

3.7 Transformation

3.7.1. Preparation of competent cells by calcium-chloride method

The strains were inoculated in 5 mL of LB medium and grown overnight at 37 ^{o}C and 200 rpm. After 16-18h, 1 mL of each culture was used to inoculate a new erlenmeyer containing 100 mL of LB, incubated at 37 ^{o}C and 200 rpm until OD_{600} reached 0.3. At this stage, the culture was transferred to a 50 mL sterile falcon and centrifuged at 7 000 xg, 4 ^{o}C for 2 min. The supernatant was discarded and the cells suspended in 50 mL of 0.1M MgCl2 solution, followed by another centrifugation step at 7 000 xg, 4 ^{o}C for 2min; then the supernatant was discarded. The pellet was suspended in 50 mL of 0.1M $CaCl_2$.The suspension was incubated 20 min on ice and centrifuged at 7 000 xg at 4 ^{o}C for 2 min again, the supernatant was discarded and the pellet suspended in 1 mL of 0.1 M $CaCl_2$ with Glycerol 15 % solution. Aliquots of 100 µL were distributed into several tubes, properly identified and stored at -80 ^{o}C until use.

3.7.2. Transformation by heat-shock

Two aliquot of 100µL of each previously prepared competent cells were thawed on ice and 5µL of the vector were added to the tube. The mixture was incubated on ice for 15 min and transferred to 42 ^{o}C for 45 sec. The tube was immediately placed on ice for 2 min and 1 mL of LB medium was added. The cells were then grown for 1 hour at 37 ^{o}C, 200 rpm; the culture was centrifuged at 5 000 rpm for 1 min to collect cells; most of supernatant was discarded and the pellet was suspended in the remaining supernatant.

Finally the cells were spread on LB agar plates containing the appropriate antibiotics and incubated at 37 ^{o}C, overnight.

3.7.3. Preparation of competent cells for electroporation

The desired strain was inoculated in 10 mL of LB medium supplemented with the appropriate selective marker and incubated at 37 ^{o}C, 180 rpm, and overnight. 1.5 mL of this overnight culture were used to inoculate 125 mL of fresh 2XYT broth containing the appropriate selective marker. The culture was grown in the same conditions until OD_{600nm} of approximately 0.4. The culture was then centrifuged at 5000 rpm for 5 min and 4 ^{o}C. The supernatant was discarded and the pelleted cells were resuspended in 30 mL of cold 10 % glycerol by gently mixing. This procedure was repeated again. After centrifugation, the cells were suspended in 1 mL of cold 10 % glycerol and distributed in 1.5 mL tubes. These competent cells were kept at 4 ^{o}C until use. This procedure was always performed in the same day of the transformation.

3.7.4 Transformation by electroporation

50 µL of cells were mixed with 5 µL of DNA and maintained on ice. The mixture was transferred to a 1 mm gap ice-cold electroporation cuvette and a single pulse was applied using the Ec1 program (200Ω, 25µF and 2.5RV) in the MicroPulser Electroporator (BioRad). Immediately, 950 µL of LB medium were added to the cuvette and the suspension was incubated at 37 ^{o}C for 1 hour at 180 rpm. The culture was then spread in LB agar plates containing the appropriated antibiotics. The plates were incubated at 37 ^{o}C overnight.

4. Results and Discussion

4.1 Selection of a white/blue system for library construction

The *trans* complementation system available for the production of lichenicidin in *E. coli* did not allow white/blue selection. This is because *E. coli* BL21Gold(DE3) possesses the *lacZ* gene in its chromosome. Additionally, the plasmid used (pET-24a) in that system did not contain this gene. Thus, in the present study, the pLic5Δ*A1A2* fosmid (comprising the entire lichenicidin gene cluster, except *licA1* and *licA2* structural genes) was transformed into 3 different *E. coli* strains: DH5α, TOP10 and Mach1T1, all of them permitting white/blue selection. Then, each of the genes *licA1* and *licA2*, was amplified and cloned in the multiple cloning site of pUC19a plasmid, disrupting the *lacZ* gene. However, it was found that the colonies of all the strains containing the p*licA2* plasmid still express the blue phenotype, which was not observed with the p*licA1* strains (**Figure 11**). Nucleotide sequences of both structural genes cloned, revealed that in *licA1* the stop codon was TAA, whereas in *licA2* it was TAG. These were the original stop codons, i.e., from *Bacillus licheniformis* I89. In *E. coli*, TAA corresponds to "ochre" codon and TAG corresponds to the "amber" codon, which results in a weaker termination. Hence, we replaced the TAG codon by TAA in the p*licA2*, originating the p*licA2*ST plasmid. After this change, the colonies presented a whiter phenotype, as expected. Therefore, it was concluded that the use of "amber" stop codons in these systems should be avoid.

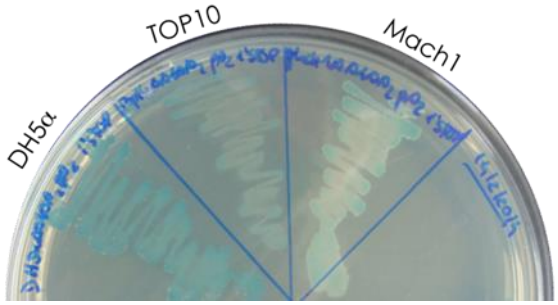

Figure 11: Colonies color when the original STOP codon is present.

Afterwards, the agar-based production of lichenicidin peptides by the three strains was evaluated by colony-bioassay at two different temperatures: 30 ºC and 37 ºC (**Figure 12 and 13**). It was found that the inhibition zones were greater at 37 ºC, for both peptides. Therefore, this was the temperature chosen for the screening of the random mutagenesis library. For Bliα, the levels of production do not seem to be influenced by the nature of the strains used, since no major differences in inhibition were observed between DH5α, TOP10 and Mach1T1 strains (**Figure 12**). For Bliβ, a comparison of the strains with p*licA2*ST (ochre stop codon) and p*licA2* (amber stop codon), a slight increase in the production can be observed for the first, especially for Mach1 and DH5α (**Figure 13**). Accordingly, any of these two *E. coli* strains can be used for the library construction. Finally, Mach1 was selected because DH5α colonies containing both pLic5Δ*A1A2* and the p*licA2*ST still exhibited a shade of blue color.

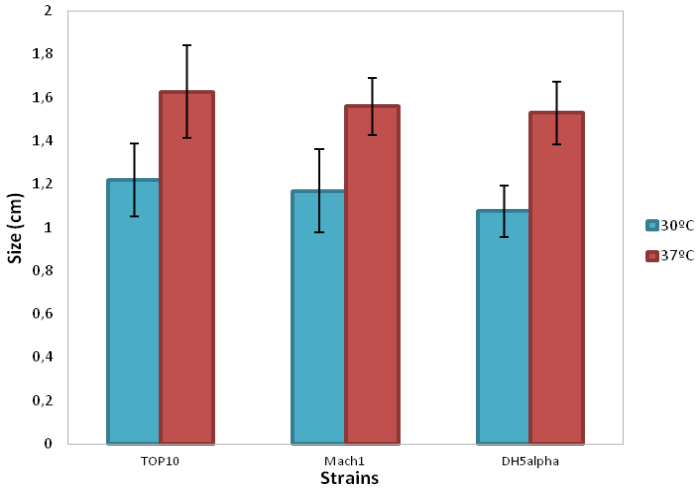

Figure 12: Radius size of colony-bioassay of the three *E.coli* strain producing Bliα at 30 ºC and 37 ºC.

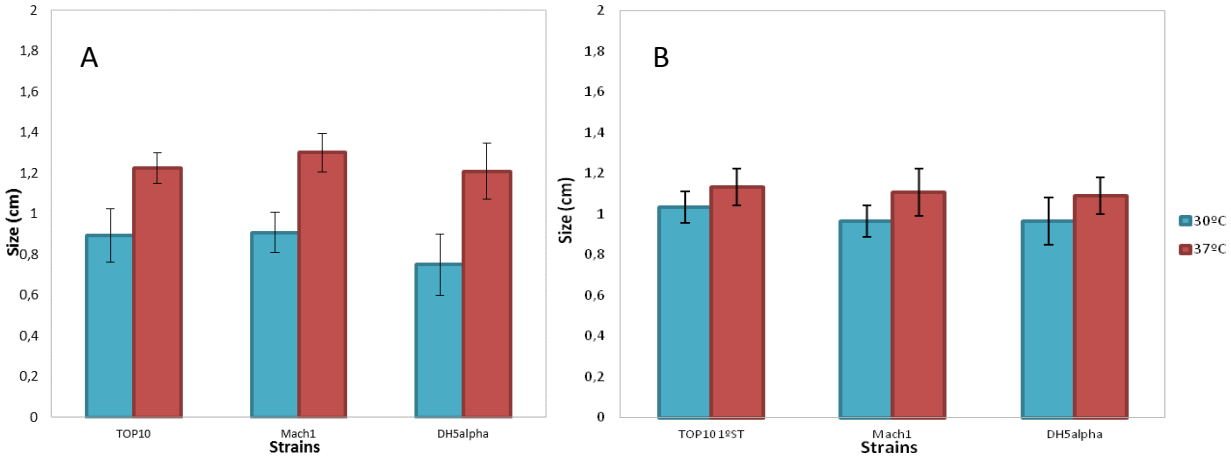

Figure 13: Radius size of colony-bioassay of three *E. coli* strains producing Bliβ at 30ºC and 37ºC, with plasmid p*licA2*ST (A) and p*licA2* (B).

4.2 Screening of the random mutagenesis library

The screening of the two libraries, was performed by colony-bioassay. Clones with different bioactivity from that of the control were selected. The clones were grouped into 4 distinct groups: null activity (NA), very low activity (VLA), reduced activity (RA) and higher activity (HA) when compared to control (**Figure 14**).

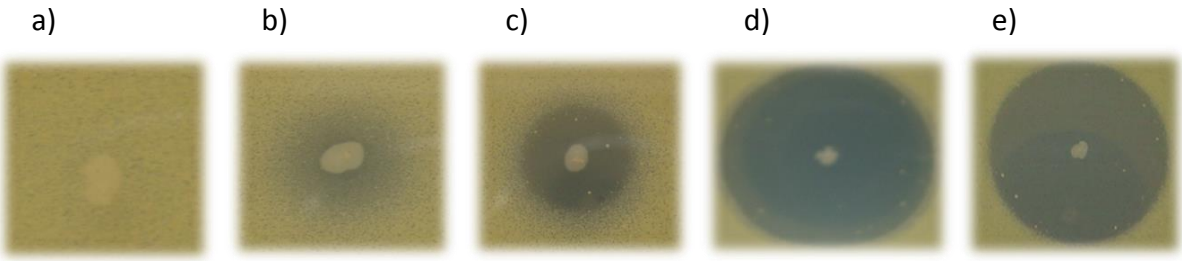

Figure 14 Different classification of the strains with the mutated genes. a) clones with null activity (NA), b) clones with very low activity (VLA), c) clones with activity reduced to 50 % comparing to the control (RA), d) clones with higher activity (HA) and e) clones with no mutated genes (control).

Considering the Blicα library, around 4.5 % of the clones showed null activity, 2.6 % present very low activity, 2.2 % reduced activity and 0.5 % higher activity. The percentage for Blicβ library was very similar since around 5.1 % clones showed null activity, 1.4 % very low activity, 3.8 % reduced activity and 0.58 % of the clones present higher activity. To confirm a high producing phenotype, clones exhibiting higher activity were tested by a dot-colony-bioassay. This method is more reliable since it is more accurate and the number of inoculated cells is identical between samples. This assay revealed that none of the clones showed improved activity.

Clones belonging to NA, RA and VLA groups were selected for sequencing of the structural gene, to determine the mutations responsible for those phenotypes. The antibacterial activity of all of these clones was confirmed prior to sequencing, by colony-bioassay for colonies with very low and null activity and by dot-colony-bioassay for mutants showing reduced activity. So, for the Bliα library, 45 clones with the NA phenotype, 30 from the RA group and 43 belonging to VLA group were analyzed. For Bliβ library, 43 colonies of the NA group, 30 from RA and 30 with VLA phenotype were analyzed. The results showed that the structural gene of numerous clones with null

activity had premature stop codons (n=15) and frameshift mutations (n=13). Since these mutations prevent the production of the encoded peptides, they will not be further discussed. Also, some structural genes (n=26) contained more than one nucleotide mutation, responsible for more than two changes in the amino acid sequence of the LicA1 and LicA2 precursor peptides. In this case, the impact on bioactivity could not be attributed to a single change and for this reason these clones will also not be considered in this study.

The mutations identified will be discussed in detail in the following sections.

4.3 Mutations in the leader sequence of Bliα

The leader peptides of Class II lantibiotics terminate with a double-Gly motif like Gly-Gly/Gly-Ala/Gly-Ser. This motif plays an essential role in the cleavage of the leader sequence since it is believed that the protease domain of the LanT protein recognizes it (Mcauli *et al.*, 2001). In this library, only two mutations were found in the leader peptide of LicA1, both affecting the double-Gly motif (**Figure 15**). In one clone, the second Gly (G44) residue of the normal GG-motif was replaced by a Cys and exhibited no bioactivity. This suggests that the protease (LicT) is unable to remove the leader sequence of the mutated peptide. This was also observed for mutacin II and lacticin 481. *In vitro* studies of these two lantibiotics, demonstrated that mutations on the double-Gly motif result in fully dehydrated and cyclized peptides, however proteolysis by LctT was completely inhibited (Ihnken *et al.*, 2008; Patton *et al.*, 2009). The other mutation identified was the substitution of the first Gly amino acid (G43) by a Glu. Interestingly, in this clone, a reduction to half of the bioactivity, in comparison to the control, was observed. This result was surprising because the G43E mutation should prevent the removal of the Bliα leader peptide and in this situation, the peptide would be inactive. Thus, further work should be performed to fully understand if the reduced activity results from less efficiency of the proteolysis reaction or differences in the post-translational modifications.

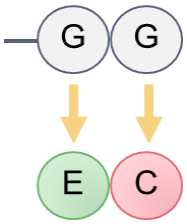

Figure 15: Mutations detected in the double-Gly motif of Bliα leader sequence. In red is the mutation which caused a total abolishment of the bioactivity. In green is the mutation which caused a reduction to half of the bioactivity when compared to the control.

4.4 Mutations in the leader sequence of Bliβ

A mutation on the double-Gly motif was also detected in the leader sequence of Bliβ, where the second Gly residue was substituted by a Val (**Figure 16**). As mentioned above for Bliα, this alteration would imply the inhibition of the leader peptide proteolysis, resulting in a completely inactive peptide. However, although very low, the clone still exhibited antibacterial activity, suggesting that albeit at very low levels, a mature peptide is being produced. Therefore, more studies will be required to elucidate if the protease domain of LicT is actually able to recognize the –GV sequence. Alternatively, it is also possible that the phenotype observed is due to the second proteolysis step involved in the maturation of Bliβ.

The other mutations identified were P21T, P21S, H20R and A22E (**Figure 16**). The mutations P21S and P21T resulted in reduction to half of the bioactivity in comparison to the control, perhaps because Pro is an essential amino acid for the peptide structure. The H20R mutation also resulted in a similar phenotype. However, in this case it was shown that the substitution of one positively charged amino acid with other with the same nature still affected the efficiency of LicM2 protein, despite at low levels. Therefore, the imidazole functional group present in the Arg amino acid should be important for the perfect recognition of the leader peptide by the LicM2 modifying enzyme. The last mutation identified was A22E. This clone was completely inactive. This was a surprising result because so far it was not described that a single mutation in the leader sequence could cause a total lack of bioactivity. The LC-ESI-MS analysis of the extracts of the A22E clone showed that the fully dehydrated Bliβ peptide was produced (M= 3020 Da). Thus, MS/MS analysis should be performed in order to understand which thioether ring was affected by this mutation and that lead to its inactivity.

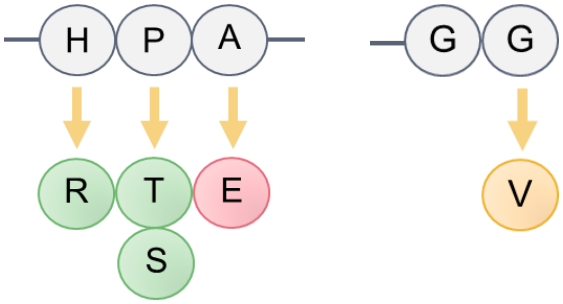

Figure 16: Mutations along of Bliβ leader sequence and in the double-Gly motif. In red is the mutation which caused a total abolishment of the bioactivity. In orange is represented a mutation that greatly affected the bioactivity and so the mutant showed very low bioactivity. In green are the mutations which cause a reduction to half of the bioactivity when compared to the control.

4.4.1 Mutations in the hexapeptide

As already mentioned, the maturation of the Bliβ peptide implies two proteolytic cleavages: i) the removal of leader peptide by LicT and ii) the removal of six N-terminally located amino acids, commonly designated by hexapeptide (in LicA2: NDVNPE) (Caetano *et al.*, 2011a). This extra proteolytic step was also identified for other two-component lantibiotics, namely, haloduracin β-peptide (Halβ), plantaricin W β-peptide (Plwβ) and cytolysin L and S peptides ($CylL_L$ and $CylL_S$) (Booth *et al.*, 1996; Holo *et al.*, 2001; McClerren *et al.*, 2006). One possible role of this six N-terminal amino acids is related to the protection of the peptide from being degraded in the extracellular environment, since proteolysis of the peptide only occurs when it is outside the cell (Cooper *et al.*, 2008). In the biosynthesis of Bliβ, the removal of the hexapeptide is performed by LicP protease (Caetano *et al.*, 2011a). Along the screening of the Bliβ mutant library, two mutations (P41H and E42K) in the hexapeptide causing the reduction of bioactivity to half were identified (**Figure 17**). These alterations involved Pro and charged residues, these amino acids are normally important either for secondary structure and/or recognition. Studies targeting the hexapeptide are limited. Still, for lichenicidin, it was previously observed that the substitutions P41A and E42A did not result in loss of bioactivity, suggesting that these residues were not essential for LicP function (Faria, 2011). Yet, Ala is a neutral amino acid whereas His and Lys are charged residues.

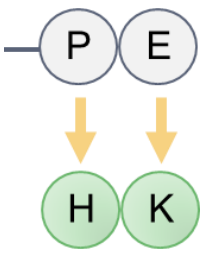

Figure 17: Mutations identified in the Hexapeptide of Bliβ. In green are the mutations which cause a reduction to half of the bioactivity when compared to the control.

4.5 Mutations detected in Bliα

Several types of mutations were identified in the core peptide of LicA1 affecting conserved residues of the α-peptides, which included changes in residues involved in the formation of Lan and MeLan rings and other amino acids. All these mutations will be discussed in the following sections.

4.5.1 Bliα mutagenesis of conserved residues

The α-peptides of two-component lantibiotic have a high homology with mersacidin. Thus, the alignment of several of these lantibiotics such as staphylococcin C55 (Navaratna *et al.*, 1999), plantaricin W (Holo *et al.*, 2001), haloduracin (McClerren *et al.*, 2006) and lacticin 3147 (Ryan *et al.*, 1996; Martin *et al.*, 2004) allowed to identify a set of conserved amino acids (**Figure 18**). The residues TX(T/S)XECX(XX)C appear to form the rings that comprise the main site for the interaction with lipid II, especially the C-ring (Cotter *et al.*, 2006).

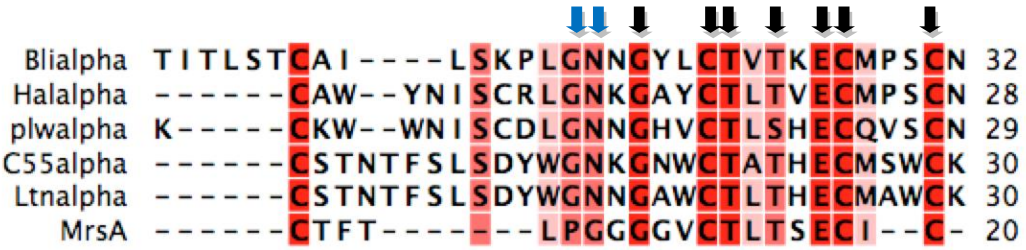

Figure 18: Alignment of α-peptides of some two-component-peptide lantibiotics and mersacidin. Black arrows indicate the amino acids conserved in mersacidin-like peptides. Blue arrows show the residues conserved among α-peptides, but not mersacidin.

Only two of the conserved amino acids of mersacidin-like peptides are not involved in the formation of Lan and/or MeLan bridges: the Gly (G18 in Bliα) and the Glu (E26 in Bliα). In the present study, a non-active mutant was identified where the E26 residue was replaced by an Asp (**Figure 20**). This was in agreement with what has been already reported, in which the alteration E26A resulted in a fully produced peptide, however its antibacterial activity was completely abolished (Caetano *et al.,* 2011a). Moreover, when the corresponding residues in mersacidin (Szekat *et al.*, 2003), Halα (Cooper *et al.*, 2008)

and Lntα (Cotter *et al.*, 2006) were substituted by Ala, the peptides produced were inactive. These results were due to the fact that this Glu is essential for the binding of the peptides to its target lipid II (Szekat *et al.*, 2003).

The LC-ESI-MS analysis of extracts of the mutant E26D of Bliα showed that the peptide is being produced, however it is also completely inactive. This result showed that the substitution of Glu is not possible in these peptides, even when the negative charge is maintained (as is the case of Asp). A modification in the G18 residue to Arg was also identified in a clone that completely lacks bioactivity (**Figure 20**). A similar mutation in the α-peptide of lacticin 3147 (G16R) also resulted in the abolishment of activity (Field *et al.*, 2013). Thus, this residue is also not tolerant to amino acid changes, being therefore essential to the bioactivity of the lantibiotics.

C21, T22, C27 and C31 are the conserved residues involved in the formation of the B-, C- and D-rings of Bliα. In our library, the following mutations were identified in these four residues: C21S, C27Y, C27F, T22I, C31S and C31R and none of these clones were able to inhibit growth of *M. luteus* (**Figure 19**). The exception was T22R substitution, which showed an inhibition zone with half of the size when compared with the control. This result was not expected because the modification of T22 by Ala, previously described by Caetano *et. al* (2011a), resulted in a peptide without antibacterial activity. Hence, it was concluded that formation of the Bliα C-ring was essential to bioactivity. The presence of an Arg residue instead of T22 prevents the formation of this ring in the mutated peptide. Nevertheless, the clone showed some bioactivity. Therefore, further studies will be required to understand the reason behind such phenotype.

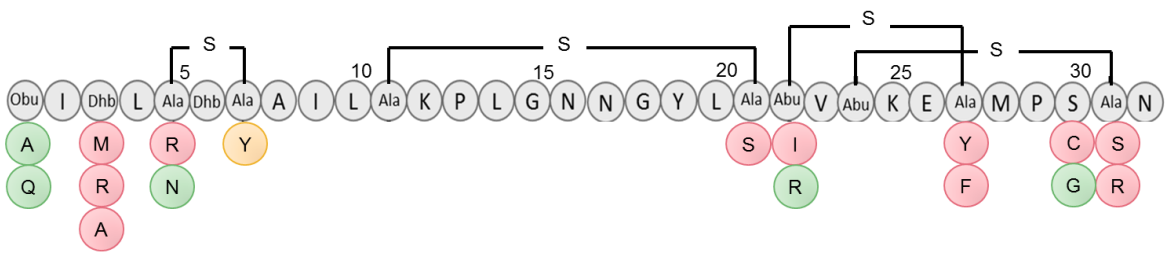

Figure 19: Changes in Ser/Thr and Cys residues in Bliα and their respective bioactivities. In red are the mutations which cause a total abolishment of the activity. In orange are represented mutations that greatly affect the bioactivity and so the mutants show a very low bioactivity. In green are the mutations which cause a reduction to half of the bioactivity when compared to the control.

There are two conserved amino acids among α-peptides, but not in mersacidin (**Figure 18**). These correspond to the G15 and N16 residues in Bliα. In this work, the mutation N16K was identified in a clone with no detectable activity (**Figure 20**). This lack of activity is not due to the absence of BliαN16K production, since the mutated peptide (M= 3264 Da) was identified by LC-ESI-MS analysis. A similar result was found in the N14 residue from Ltnα when it is substituted by Ala, highlighting the importance of this residue among α-peptides of the two-component lantibiotics (Cotter *et al.*, 2006).

4.5.2 Mutations in other Cys, Ser and Thr residues of Bliα

In this study, 6 from a total of 10 mutants with changes in amino acids involved in the formation of Bliα Lan and MeLan tioether rings, showed no activity towards *M. luteus*. This was not surprising since previous studies on other class II lantibiotics demonstrated that changes in these residues resulted in a decrease or a total loss of bioactivity (Ottenwälder *et al.*, 1995; Bierbaum *et al.*, 1996). The proposed residues involved in the two Lan and the two MeLan rings in Bliα are: S5 and C7 form the A-ring, S11 and C21 to the B-ring, T22 and C27 for C-ring and T24 and C31 for D-ring (Begley *et al.*, 2009; Caetano *et al.*, 2011a). The mutations involved in the B-, C- and D-rings were discussed in the previous sections because they are part of the conserved motifs of the mersacidin-like lantibiotics. Regarding the A-ring, the following mutations were detected: S5R, S5N and C7Y (**Figure 19**). The outcome of these alterations, in terms of bioactivity, was quite variable: S5R was inactive and S5N showed a reduction to half of bioactivity when compared to the control. The clone with the C7Y modification presented a very low activity against *M. luteus*. When S5 and C7 residues were independently substituted by Ala, the bioactivity was reduced to half (Caetano *et al.*, 2011a) . Thus, our results also confirm that the A-ring is not as important as the other rings for the antibacterial activity of Bliα. Nevertheless, mutations altering the charge of the residues (such as S5R) are not tolerated.

Bliα peptide possesses 7 Ser and Thr residues that are posttranslationally dehydrated and one Ser (S30) that escapes this modification (Begley *et al.*, 2009). In the present

study, two different mutations in this Ser residue were identified: S30G and S30C (**Figure 19**). The S30G mutant showed a reduction to half of the antimicrobial activity in comparison to the control. This result is in agreement with the documented S30A modification, in which bioactivity was decreased but not abolished (Caetano *et al.*, 2011a). However, the substitution of S30 by a Cys resulted in a clone without antibacterial activity. An explanation for this can be the fact that one Cys in that position may interfere with the correct formation of the D-ring, which is a very important ring for bioactivity. For instance, when some residues of Nisin and Pep5 were modified to Cys, a decrease on the activity was also observed (Cotter et al. 2005b).

The residues Thr3 and Thr6 are both dehydrated to Dhb in the mature Bliα. In this study, the following mutations were detected in the T3 residue: T3A, T3M and T3R. All of these changes resulted in total lack of bioactivity (**Figure 19**). These results are not in accordance with others where the same mutation (T3A) was studied, but where only a reduction to half of the bioactivity was observed (Caetano *et al.*, 2011a). Therefore, this result should be further confirmed. In the literature, it has been referred that the consequence of mutating Dha and Dhb residues probably depends on the location of the residue within the peptide (Cotter et al. 2005b). When Dhb residues in Pep5 were replace by Ala (T16A and T20A), a significant loss of activity was also observed. However, when the Dhb in nisin Z was changed to Ala and Val (T2A and T2V), the clones displayed either wild-type or close to wild-type levels of activity (Cotter *et al.*, 2005b).

As previously described, after proteolysis of the leader sequence, a spontaneous deamination of the Dhb1 to a 2-oxobutyryl (Obu1) occurs in Bliα. Herein, two mutations were identified in this residue: T1A and T1Q. Both resulted in a decrease to half of the bioactivity (**Figure 19**), demonstrating that this residue is not very important to Bliα peptide activity, as previously suggested by Caetano *et al.* (2011a).

4.5.3 Mutations in other residues of Bliα

Some clones possessing mutations not involving conserved or other Ser and Thr residues were identified in this study. One such mutation is Y19C, which was previously associated with very low activity (**Figure 20**). Tyr is an aromatic amino acid and this type of residues have membrane-seeking properties (Cotter *et al.*, 2006). Additionally, and more importantly, the insertion of an extra Cys in the core peptide of Bliα will most probably disturb the correct formation of Lan/MeLan rings. Nevertheless, it will be interesting to establish if in this situation, 5 rings will be formed instead of the four normally occurring. Other mutations in residues located inside of the B- and C-ring were detected, including P13A, P13L, N16K, G18R, L20P, L20Q, V23E and P29S. The analysis of the antibacterial activity of these mutants demonstrated, once more, that introduction of charged amino acids instead of neutral residues should be avoided (N16K, G18R and V23E) (**Figure 20**). Pro is an amino acid important at the structural level. However, the significance of these residues seems difficult to predict. For instance, the substitution of P13 by a Leu results in a peptide that can be identified by LC-ESI-MS analysis, but is inactive. Yet, altering the same residue to an Ala results in a peptide that is still active. This same phenotype was obtained in a clone possessing a Ser instead of P29 amino acid. Also, an inactive peptide was produced when a Pro was inserted in the position 20, instead of a Leu. Still, the L20Q mutation resulted in a active peptide (half bioactivity). Thus, our results suggest that the chemical nature of the substitution is critical for the bioactivity. Other three mutations were found in the region between the A- and the B-ring, which were A8V, A8T and I9N. Regarding the two mutations in the residue A8, only a reduction of the bioactivity was detected, showing that this position can be tolerant to changes in amino acids. The clone possessing the I9N alteration produced a small inhibition area. However, this outcome is most probably associated with the introduction of a positively charged amino acid (Arg) than with the functionality of the Ile residue (**Figure 20**).

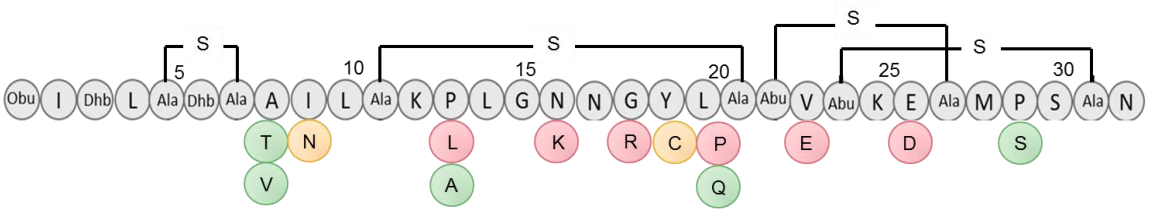

Figure 20: Bliα mutations found not in Ser/Thr and Cys residues and their respective bioactivities. In red are the mutations which cause a total abolishment of the bioactivity. In orange are represented mutations that greatly affect the bioactivity and so the mutants show a very low bioactivity. In green are the mutations which cause a reduction to half of the bioactivity when compared to the control.

4.6 Mutations detected in Bliβ

Like in Bliα, several types of mutations were identified in the core peptide of LicA2 affecting conserved residues of the β-peptide, which included changes in residues involved in the formation of Lan and MeLan bridges and other amino acids. All these mutations will be discussed in the following sections.

4.6.1 Bliβ mutagenesis of conserved residues

For β-peptides, there is not a representative like mersacidin for α-peptides. However, the alignment of several β-peptides of two-component lantibiotics reveals the presence of conserved residues, especially at their C-terminal (**Figure 21**). These constitute the core motif SXXXCPTT(K/A)C(T/S)XXC (Cotter *et al.* 2006; Cotter *et al.* 2005b). It has been suggested that this conserved motif might have an important role in the mode of action of lacticin 3147, specifically for the interaction of Ltnβ with Ltnα (Cotter *et al.*, 2006).

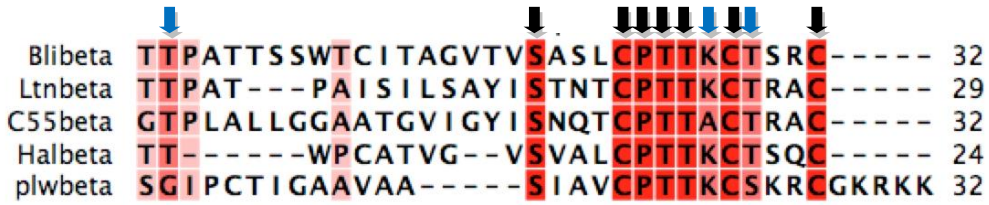

Figure 21: Alignment of β-peptides of some two-component lantibiotics. Black arrows indicate the amino acids conserved in all the peptides. Blue arrows show other highly conserved amino acids.

Considering these conserved residues, mutations on the C23, T25, C28, T29 and C32 amino acids of Bliβ were identified in clones without bioactivity (**Figure 22**). All of these amino acids are involved in bridge formation: the C23 forms the B-ring with S19, C28 forms the C-ring with T25 and C32 forms the D-ring with T29. Thus, these results confirm that the formation of these three rings should be completely essential either for bioactivity or production of the fully maturated peptide. For instance, it was found that the mutated BliβT25A peptide was not produced and therefore no antibacterial activity could be associated to it (Caetano *et al.*, 2011a). These same conclusions were inferred from studies involving the random mutagenesis of Ltnβ peptide (Cotter *et al.*, 2006).

The T26 residue of Bliβ is the only amino acid of the conserved motif that is not involved in the formation of thioether bridges. Also, it is one of the Thr residues that is dehydrated in Bliβ (Begley *et al.*, 2009; Caetano *et al.*, 2011a). In the present study, two mutations were found for T26 (**Figure 22**). While T26A mutation showed a reduction to half of the activity when compared with the control. The T26I mutation resulted in a greater loss of bioactivity. This result suggests that despite the high degree of conservation of this residue among β-peptides, it does not necessarily indicate that it absolutely essential.

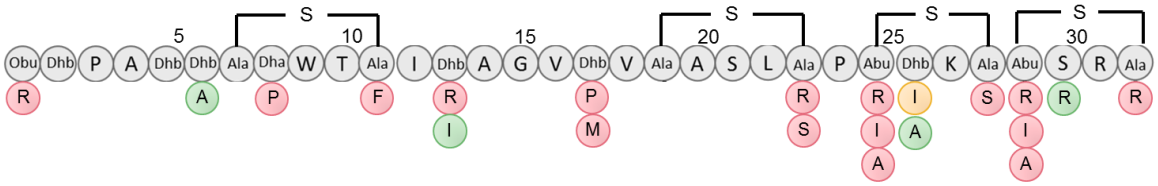

Figure 22: Bliβ mutations found in conserved residues of β-peptides and other Ser, Thr and Cys residues with their respective bioactivities. In red are the mutations that caused a total abolishment of the bioactivity. In orange are represented mutations that greatly affect the bioactivity and so the mutants show a very low bioactivity. In green are the mutations that caused a reduction to half of the bioactivity when compared to the control.

4.6.2 Mutations in other Ser, Thr and Cys residues of Bliβ

None of the residues involved in the A-ring formation are conserved among the β-peptides of two-component lantibiotics, suggesting that this Lan bridge is not essential for bioactivity. However, in the present study, one of the inactive clones possessed a mutation in the Cys of A-ring to a Phe (C11F; **Figure 22**). Yet, its alteration by an Ala

caused only reduction in bioactivity (Caetano *et al.*, 2011a). Moreover, in its homologous Halβ, the A-ring was considered dispensable for bioactivity. Thus, the absence of the A-ring together with a presence of an amino acid containing an aromatic ring should be a dramatic structural change for the peptide. Thus, it will be interesting to analyze if this peptide is produced and this will be done in a due time.

In Bliβ, there are three residues (S21, T26 and S30) that are not dehydrated by the cyclase-dehydratase LicM2. It is common over the lantibiotics that Ser/Thr residues escape dehydration. However, it is not clear how synthetases select these residues. Nevertheless, some authors consider that these amino acids have a key role in the biological activity of the final compound (Rink *et al.*, 2005; Cooper *et al.*, 2008). Considering the three non-hydrated, mutations were only detected for Ser30 and T26 (**Figure 22**). The S30R mutant retained half of the bioactivity, when compared with the control. A similar result was also obtained, for S30A substitution (Caetano *et al.*, 2011a). Thus, this residue is important for bioactivity, but not essential. T26 mutations have been discussed above.

Among the other Ser and Thr residues that are dehydrated, mutations were identified only for T1, T6, S8, T13 and T17 (**Figure 22**). Most of these involved substitutions with charged amino acids or Pro (T1R, S8P, T13R and T17P) in which the antibacterial activity was abolished. Yet, other mutation in T17 was detected (T17M) where the bioactivity was nonexistent, suggesting that this residue (T17) is not very tolerant to changes. Regarding T13, was detected other modification (T13I), where the bioactivity was only reduced. The same result was obtained for T13A mutation (Caetano *et al.*, 2011a). Thus, the phenotype of T13R should be more related with the presence of a positive charge than with the functionality of the original amino acid. The last mutation of a dehydrated amino acid was T6A. The result obtained in this study for T6A was absolutely in agreement with the alanine scan performed previously by Caetano *et al*. (2011a).

4.6.3 Mutations in other residues of Bliβ

The mutations identified in this study involving other residues of the Bliβ peptide were limited to the amino acids P3, A4, W9, I12, G15, V18, A20 and K27 (**Figure 23**). Once more, the introduction of negatively charged amino acids implicated always the absence of bioactivity (G15D, V18D, A20D and K27E). Thus, like in Bliα, when charged amino acids are introduced, a big impact on the bioactivity was observed. However, the K27M mutation had not a so detrimental effect on activity as K27E. Substitutions with Cys were also associated with very low or loss of activity (W9C and G15C). This is also understandable because lantibiotics known so far do not have free Cys residues. Therefore, insertion of an additional Cys in the core peptide will most probably result in formation of a novel ring or will disturb the normal ring-topology.

Other mutations detected that resulted in a reduction of bioactivity were P3H and A4P. The latter involves the presence of an additional Pro in Bliβ. Thus, as observed for Bliα, the replacement of a given residue by a Pro or the introduction of a Pro, have a negative impact in the antimicrobial activity. The first (P3H) will imply the loss of the first Pro in Bliβ and the presence of a positive charge (His). I12T is another mutation found that resulted in very low activity, maybe because the insertion of an additional Thr residue can alter the dehydration pattern of the entire peptide. This should be further investigated through the analysis of the MS/MS pattern of the produced peptide.

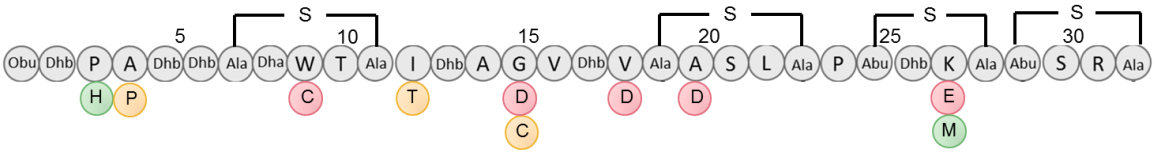

Figure 23: Bliβ mutations found in other residues and their respective bioactivities. In red are the mutations that caused a total abolishment of the bioactivity. In orange are represented mutations that greatly affect the bioactivity and so the mutants show a very low bioactivity. In green are the mutations which cause a reduction to half of the bioactivity when compared to the control.

5. Conclusions and Future Perspectives

Studies involving the bioengineering of lantibiotics to produce new variants of these peptides have been increasing in the last years. One of the main goals has been the identification of peptides with improved activity. However, this has been proven difficult. Therefore, bioengineering studies are also often used to provide information on the structure-function relationship of lantibiotics.

Among the two-component lantibiotics, random mutagenesis libraries was obtained only for lacticin 3147. The approach used in the study described by Field *et al.* (2007) implied the generation of only one library, where the mutagenesis of the lacticin 3147 peptides was performed simultaneously. Though, the antibacterial activity of two-component lantibiotics depends on the synergy between the two peptides. Thus, in this study, the impact of mutations in the bioactivity of lichenicidin peptides (Bliα and Bliβ) was investigated by mutagenesis of each peptide separately. Consequently, the complementary peptide was always provided in its wild-type form in the bioactivity assays. Therefore, two random mutagenesis libraries for lichenicidin were produced and the antibacterial activity of around 4000 clones from each library was analyzed.

Herein, it was not possible to identify mutants with improved activity against *M. luteus*. In fact, the identification of lantibiotics with this characteristic has been difficult. One successful example was nisin, a class 1 lantibiotic, where a mutant generated by random mutagenesis exhibited enhanced efficacy against both Gram-positive and Gram-negative bacteria (Field *et al.*, 2012). Yet, one of the objectives was also to recognize mutations in lichenicidin peptides resulting in reduced or null activity. This would retrieve more information on the structure-activity of lichenicidin. Moreover, interesting mutants can also be used in studies of their mode of action. Indeed, it was possible to identify several mutations related with the reduction of bioactivity. The majority of them were found in the core peptide of LicA1 and LicA2, suggesting that single mutations in the leader sequence do not have much impact in biosynthesis. An exception to this was an inactive clone identified in the Bliβ library possessing the A22E mutation in the leader sequence. Other interesting mutations detected in this study were in the

double-Gly motif. The G43E (Bliα) and G36V (Bliβ) variants still retained some bioactivity. This was not expected, since they would prevent the proteolysis of the leader sequence. Therefore, the clones with these three mutations should be further studied in detail in order to clarify their real impact at the biosynthetic level.

Considering the core peptide, several mutations on residues involved in conserved domains or in the formation of rings (especially on the C-terminal) were identified. Substitutions involving charged amino acids or prolines were also detected. Therefore, our study showed that the bioengineering strategies for these peptides should focus on their N-terminal and avoid the use of charged amino acids and prolines. Previously, the importance of the Glu26 of Bliα, and its counterparts in class II lantibiotics, was already established. However, in our study, it was shown that its most similar amino acid (Asp) is not able to play the same role as Glu26 in the mode of action of lichenicidin. Therefore, it could be interesting to compare this derivative of Bliα with the wild-type in studies involving the interaction with lipid II. Regarding the Bliβ variants herein studied, it will be necessary to perform LC-MS analysis studies in order to understand if some of the inactive variants herein identified are produced.

In the future, the nucleotide sequence of more mutants with reduced activity should be performed and always supplemented with LC-ESI-MS and LC-ESI-MS/MS analysis. Moreover, it will also be interesting the study of mutations occurring in clones that showed no altered activity.

Bibliography

Arnison PG, Bibb MJ, Bierbaum G, *et al.* (2013) Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Nat Prod Rep* **30**: 108–160.

Begley M, Cotter PD, Hill C & Ross RP (2009) Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for LanM proteins. *Appl Environ Microbiol* **75**: 5451–5460.

Bierbaum G & Sahl H-G (2009) Lantibiotics: mode of action, biosynthesis and bioengineering. *Curr Pharm Biotechnol* **10**: 2–18.

Bierbaum G, Szekat C, Josten M, Heidrich C, Kempter C, Jung G & Sahl HG (1996) Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5. *Appl Environ Microbiol* **62**: 385–392.

Booth MC, Bogie CP, Sahl H-G, Siezen RJ, Hatter KL & Gilmore MS (1996) Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. *Mol Microbiol* **21**: 1175–1184.

Brakmann S & Schwienhorst A (2004) *Evolutionary Methods in Biotechnology*. Schwienhorst SB and A, ed. betz-druck gmbh, Darmstadt, Federal Republic of Germany.

Caetano T (2011) Biossíntese da lichenicidina e pesquisa de novos péptidos antibacterianos Lichenicidin biosynthesis and search for novel antibacterial peptides. University of Aveiro.

Caetano T, Krawczyk MJ, Mösker E, Süssmuth RD & Mendo S (2011a) Heterologous expression, biosynthesis, and mutagenesis of type II lantibiotics from *Bacillus licheniformis* in *Escherichia coli*. *Chem Biol* **18**: 90–100.

Caetano T, Krawczyk JM, Mösker E, Süssmuth RD & Mendo S (2011b) Lichenicidin biosynthesis in *Escherichia coli*: licFGEHI immunity genes are not essential for lantibiotic production or self-protection. *Appl Environ Microbiol* **77**: 5023–5026.

Chatterjee C, Paul M, Xie L & van der Donk W a (2005) Biosynthesis and mode of action of lantibiotics. *Chem Rev* **105**: 633–684.

Cooper LE, McClerren AL, Chary A & van der Donk WA (2008) Structure-activity relationship studies of the two-component lantibiotic haloduracin. *Chem Biol* **15**: 1035–1045.

Cotter PD, Hill C & Ross RP (2005a) Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol* **3**: 777–788.

Cotter, D P, Hill C & Ross RP (2005b) Bacterial lantibiotics: strategies to improve therapeutic potential. *Curr Protein Pept Sci* **6**: 61–75.

Cotter PD, Deegan LH, Lawton EM, Draper LA, O'Connor PM, Hill C & Ross RP (2006) Complete alanine scanning of the two-component lantibiotic lacticin 3147: generating a blueprint for rational drug design. *Mol Microbiol* **62**: 735–747.

Dischinger J, Josten M, Szekat C, Sahl H-G & Bierbaum G (2009) Production of the novel two-peptide lantibiotic lichenicidin by *Bacillus licheniformis* DSM 13. *PLoS One* **4**: e6788.

Draper L a, Ross RP, Hill C & Cotter PD (2008) Lantibiotic immunity. *Curr Protein Pept Sci* **9**: 39–49.

Faria R (2011) Lichenicidin biosynthesis: LicP protease specificity. Universida de Aveiro.

Field D, Collins B, Cotter PD, Hill C & Ross RP (2007) A system for the random mutagenesis of the two-peptide lantibiotic lacticin 3147: analysis of mutants producing reduced antibacterial activities. *J Mol Microbiol Biotechnol* **13**: 226–234.

Field D, Connor PMO, Cotter PD, Hill C & Ross RP (2008) The generation of nisin variants with enhanced activity against specific gram-positive pathogens. *Mol Microbiol* **69**: 218–230.

Field D, Hill C, Cotter PD & Ross RP (2010) The dawning of a "Golden era" in lantibiotic bioengineering. *Mol Microbiol* **78**: 1077–1087.

Field D, Begley M, O'Connor;PM, Daly K, Hugenholtz F, Cotter P, Hill C & Ross R (2012) Bioengineered Nisin A Derivatives with Enhanced Activity against Both Gram Positive and Gram Negative Pathogens. *PLoS One*.

Field D, Molloy EM, Iancu C, Draper LA, O' Connor PM, Cotter PD, Hill C & Ross RP (2013) Saturation mutagenesis of selected residues of the α-peptide of the lantibiotic lacticin 3147 yields a derivative with enhanced antimicrobial activity. *Microb Biotechnol* **6**: 564–575.

Goto Y, Li B, Claesen J, Shi Y, Bibb MJ & van der Donk W a (2010) Discovery of unique lanthionine synthetases reveals new mechanistic and evolutionary insights. *PLoS Biol* **8**: e1000339.

Holo H, Jeknic Z, Daeschel M, Stevanovic S & Nes IF (2001) Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. *Microbiology* **147**: 643–651.

Ihnken F, A L, Chatterjee C & van der Donk WA (2008) In vitro reconstitution and substrate specificity of a lantibiotic protease. *Biochemistry* **47**: 7352–7363.

Klaenhammer TR (1988) Bacteriocins of lactic acid bacteria. *Biochimie* **70**: 337–349.

Knerr PJ & van der Donk WA (2012) Discovery, biosynthesis, and engineering of lantipeptides. *Annu Rev Biochem* **81**: 479–505.

Labrou NE (2010) Random mutagenesis methods for in vitro directed enzyme evolution. *Curr Protein Pept Sci* **11**: 91–100.

Martin NI, Sprules T, Carpenter MR, Cotter PD, Hill C, Ross RP & Vederas JC (2004) Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity. *Biochemistry* **43**: 3049–3056.

Mcauli, Olivia, Ross RP & Hill C (2001) Lantibiotics : structure , biosynthesis and mode of action. *FEMS Microbiol Rev* **25**: 285–308.

McClerren AL, Cooper LE, Quan C, Thomas PM, Kelleher NL & van der Donk WA (2006) Discovery and in vitro biosynthesis of haloduracin, a two-component lantibiotic. *Proc Natl Acad Sci U S A* **103**: 17243–17248.

Mendo S, Faustino NA, Sarmento AC, Amado F & Moir AJG (2004) Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain. *Biotechnol Lett* **26**: 115–119.

Navaratna, B. MAD, Sahl H-G & Tagg JR (1999) Identification of Genes Encoding Two-Component Lantibiotic Production in Staphylococcus aureus C55 and Other Phage Group II *S. aureus* Strains and Demonstration of an Association with the Exfoliative Toxin B Gene. *Infect Immun* **67**: 4268–4271.

Nes IF, Yoon S-S & Dzung BD (2007a) Ribosomally synthesized antimicrobial Peptides (Bacteriocins) in Lactic Acid Bacteria: A Review. *Food Sci Biotechnol* **16**: 675–690.

Nes IF, Diep DB & Holo H (2007b) Bacteriocin diversity in *Streptococcus* and *Enterococcus*. *J Bacteriol* **189**: 1189–1198.

Ottenwälder B, Kupke T, Brecht S, Gnau V, Metzger J, Jung G & Götz F (1995) Isolation and characterization of genetically engineered gallidermin and epidermin analogs. *Appl Environ Microbiol* **61**: 3894–3903.

Patton G, C R, Paul M, Cooper LE, Chatterjee C & Van WA (2009) The Importance of the Leader Sequence for Directing Lanthionine Formation in Lacticin 481. *NIH-PA Author Manuscr Biochem Author Manuscr* **47**: 7342–7351.

Rasila, S T, Pajunen IM & Savilahti H (2009) Critical evaluation of random mutagenesis by error-prone polymerase chain reaction protocols, *Escherichia coli* mutator strain, and hydroxylamine treatment. *Anal Biochem* **388**: 71–80.

Rea MC, Ross RP, Cotter PD & Hill C (2011) Prokaryotic Antimicrobial Peptides. *Prokaryotic Antimicrobial Peptides: From Genes to Applications*, (Drider D & Rebuffat S, eds), pp. 29–53. Springer New York, New York, NY.

Riley M a (1998) Molecular mechanisms of bacteriocin evolution. *Annu Rev Genet* **32**: 255–278.

Riley M a & Wertz JE (2002) Bacteriocins: evolution, ecology, and application. *Annu Rev Microbiol* **56**: 117–137.

Rink R, Kuipers A, de Boef E, Leenhouts KJ, Driessen AJM, Moll GN & Kuipers OP (2005) Lantibiotic structures as guidelines for the design of peptides that can be modified by lantibiotic enzymes. *Biochemistry* **44**: 8873–8882.

Ryan MP, Rea MC, Hill C & Ross RP (1996) An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Appl Environ Microbiol* **62**: 612–619.

Sahl HG & Bierbaum G (1998) Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annu Rev Microbiol* **52**: 41–79.

Sahl H-G, Jack RW & Bierbaum G (2008) Biosynthesis and Biological Activities of Lantibiotics with Unique Post-Translational Modifications. *Eur J Biochem* **230**: 827–853.

Shenkarev ZO, Finkina EI, Nurmukhamedova EK, *et al.* (2010) Isolation, structure elucidation, and synergistic antibacterial activity of a novel two-component lantibiotic lichenicidin from *Bacillus licheniformis* VK21. *Biochemistry* **49**: 6462–6472.

Szekat C, Jack RW, Skutlarek D, Färber H & Bierbaum G (2003) Construction of an expression system for site-directed mutagenesis of the lantibiotic mersacidin. *Appl Environ Microbiol* **69**: 3777–3783.

Vanhercke T, Ampe C, Tirry L & Denolf P (2005) Reducing mutational bias in random protein libraries. *Anal Biochem* **339**: 9–14.

Wang T, Zhu H, Ma X, Zhang T, Ma Y & Wei D (2006) Methods for Mutant Library Construction Casting a Wider Net. *Mol Biotechnol* **34**.

Wiedemann I, Böttiger T, Bonelli RR, *et al.* (2006) The mode of action of the lantibiotic lacticin 3147--a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. *Mol Microbiol* **61**: 285–296.

Willey JM & van der Donk WA (2007) Lantibiotics: peptides of diverse structure and function. *Annu Rev Microbiol* **61**: 477–501.

Appendix 1 – PCR using Promega *Taq* DNA polymerase and Electrophoresis gel

PCR using Promega *Taq* DNA polymerase

For all the reactions in order to minimize pipetting errors, a PCR master mix was performed (PCR master mix content – water, buffer, primers, dNTPs and Promega *Taq* NA polymerase). All solutions were gently vortex and briefly centrifuged (**Table 8**).

Table 8: PCR reaction using Promega *Taq* DNA polymerase

Component of the reaction	*Volume*
5X *Taq* DNA Buffer	5µL
dNTP Mix, 10mM each	0.5 µL
Forward primer (10mM)	0.75 µL
Reverse primer (10mM)	0.75 µL
Taq DNA polymerase (5U/ µL)	0.125 µL
DNA template *	1 µL
Destilled water	Up to 25 µL

*To perform colony-PCR, instead of the DNA solution as template, one isolated colony is picked to the mixture. The required final volume is performed with distilled water.

The mixture was then divided into PCR tubes and one single colony or the regular volume is added into the solution. The reactions were then placed in the thermocycler (BIO-RAD) and the following thermal cycling conditions were used (**Table 9**)

Table 9: Thermal cycling conditions to perform the PCR with *Taq* DNA polymerase from Promega.

	Temperature (ºC)	**Time**	**Number of cycles**
Initial denaturation	95	3 min	1
Denaturation	95	30seg	
Annealing	Tm-5*	30 seg	30
Extension	72	1min/kb	
Final extension	72	10 min	1

*Annealing temperature based on the average of the primers melting temperatures, which was decreased by 5 degrees.

After the amplification reaction the PCR product was stored at -20 ^{o}C until further use or immediately run in an electrophoresis gel.

Electrophoresis gel

Analysis of PCR products was generally performed on agarose gel electrophoresis. The samples were mixed with 6X loading buffer (6X loading buffer composition: 2.5 mg/mL of bromophenol blue, 2,5 mg/mL of xylene cyanol FF and 30 % (v/v) glycerol; stored at 4 ^{o}C.) in a proportion of 1:6 (v/v) and loaded in a 1 % agarose gel. The gel was prepared with 1X of TAE buffer (Bio-Rad) and EtBr (AppliChem) to a final concentration of 0.5 µg/mL added before placing the melted agarose in the running tray. In all gels 0.5 µg of DNA Ladder Mix (Fermentas) marker was included. Electrophoresis was generally performed at 120 V for the desired time and the DNA was analyzed under UV light and the image acquired in the ATTO image acquisition system.

Appendix 2 - Purification of PCR products, restriction digestions and agarose gels

Purification of PCR products and DNA digestions were performed using the NZYGelpure (NZYtech) kit, according to the manufacturer's instructions.

(1) Purification of PCR proucts and restiction digestions

The volume of the reaction mixture was transferred to a 1.5 mL microcentrifuge tube and five volumes of Binding Buffer were added and mixed well. The mixture was applied to an NZYTech spin column, incubated at room temperature for 2 min and centrifuged for 1 min at top speed. The flow-through was discarded and 600 µL of Wash Buffer were added to the spin column. After 2 min of room temperature incubation, the column was centrifuge for 1 min and the flow-through was discarded. An additional 1 min centrifugation was performed to remove residual ethanol. The NZYTech spin column was then placed into a clean 1.5 mL microcentrifuge tube and 30 to 50 µL of sterile distilled water were added to the center of the column. The DNA-containing column was incubated at room temperature for 2 min and then centrifuged for 1 min to elute the DNA. The sample was stored at -20 ^{o}C until further use.

(2) Purification of DNA from agarose gel

Regarding the NZYGelpure kit manufacture's instructions. Carefully, the desired DNA fragment was excised from the agarose gel with a clean scalpel and placed in a 1.5 mL microcentrifuge tube. The gel slice was weighted and 300 µL of Binding Buffer for each 100 mg of gel weight was added. The tube was incubated at 60 ^{o}C for 10 minutes and occasionally stirred until agarose is completely dissolved. 1 gel volume of isopropanol was added and mixed by pipetting several times. The sample was then applied to a NZYTech spin column that is placed intro a Collection tube (2 ml) and centrifuged for 1 minute and the flow-through was discarded. And the column was placed back to the collection tube. The column was washed with 500 µL of Wash Buffer and centrifuged for one minute. The flow-through was discharged and one other washed step was performed

adding 600 µL of Wash Buffer and again the column was centrifuged for one minute and the flow-through was discarded and the column was centrifuged for an additional minute to ensure the complete removal of residual ethanol. The NZYTech spin column, was placed in a clean 1.5 mL microcentrifuge tube and the DNA was eluted in 30 to 50 µL of sterile Elution Buffer. The elution is performed after 2 min of incubation at room temperature by centrifugation for 2 min at top speed. The sample was stored at -20 ^{o}C until further use.

Appendix 3 - DNA concentration – Qubit® (Invitrogen)

In order to quantify the DNA amount of the sample, a master mix was performed in a 1.5 mL tube and for each sample 199 µL of Qubit® dsDNA HS Assay buffer was added and 1 µL of DNA standard was also added to the final master mix. The master mix was vortex and suffer a fast spin step. In the next step, 199 µL of the master mix was introduced into a small tube for the Qubit® and 1 µL of DNA from the sample was also added. The tubes were vortex for 2-3 seconds, incubated at room temperature for 2 min and the concentration was read in the Qubit® Flurometer.

Appendix 4 - Plasmid and Fosmid extraction

(1) Plasmid extraction with EzWay^{TM}Plasmid DNA Kit (Komabiotech)

The routine extraction of plasmid DNA from *E. coli* was performed EzWay^{TM}Plasmid DNA Kit (Komabiotech) according to manufacturer's instructions. A bacteria culture with the desired plasmid was growth overnight in the LB medium supplemented with the respective selective markers, at 37 ^{o}C and 180 rpm. 3 ml of well-grown bacteria culture was transferred to a microcentrifuge tube and the bacteria was descended by a centrifugation step for 1-2 min. The supernatant was discarded and the pellet was suspended by pipetting in 250 µl of Resuspension Buffer. 250 µl of Lysis Buffer was added and the tube was gently inverted by 5 times (without vortexing) to lyse the cells and was incubated at room temperature for 2 min. Neutralization was performed by adding 350 µl of Neutralization Buffer and by immediately mixing by inverting the tube 5 times. The lysate was centrifuged for 10 min at maximum speed and the supernatant was transferred to a DNA-prep Column. After centrifugation for 1 min the flow-through was discarded, the column was placed in the same collection tube and 400 µl of Wash-Buffer A was added into the column. The column was centrifuged for 1 min, the flow-through was discarded and the column was washed again with 750 µl of Wash-Buffer B (with ethanol added). The column was centrifuged again for 1 min, the flow-through was discarded and the column was additional centrifuged for 3 min in order to remove residual ethanol. Finally the column was transferred to a sterile 1.5 microcentrifuge tube and the elution of the plasmid DNA was performed by adding 40 µl of Elution Buffer into the center of the membrane column, incubation for 1 min at room temperature and centrifugation at maximum speed for 1 min.

(2) Fosmid extraction with traditional alkaline lysis

To extract fosmid DNA, columns cannot be used because of DNA large size. However, the protocol can be performed using the reagents from the QIAprep Spin MiniPrep Kit (QIAGEN).

In order to extract the fosmid, the bacterial strain was grown overnight in LB medium with the appropriate antibiotic and 10 mL of the bacterial culture was centrifuged for 1 min at top speed in a to-table centrifuge. The supernatant was discarded and the cell pellet was resuspended in 250 µL of buffer P1 at 4 ^{o}C. The cell lysis was performed by the addition of 250 µL of lysis buffer (P2) and mixing, followed by the addition of 350 µL of P3 buffer. The mixture was centrifuged for 5 min at maximum speed and the supernatant was transferred to a clean 1.5 mL microcentrifuge tube. 1 volume of phenol:CIA was added and mixed well. Another centrifugation was performed in the same conditions as mentioned before. The aqueous upper phase was collected to a new microcentrifuge tube. 1/10 volume of 0.3 M of NaAc (pH 5.2) and 0.6 volume of isopropanol were then added to the recovered supernatant and the mix was incubated at room temperature for 15 min followed by a centrifugation at 4 ^{o}C, top speed for 15 min. The white pellet formed was washed with 1 mL of 70 % (v/v) ethanol and centrifuged 5 min at maximum speed. After removal of ethanol, the pellet was air-dried to remove residual ethanol. Finally the pellet was resuspended in 30 µL of sterile distilled water.

Appendix 5 - Clones and respective mutations and bioactivity of Bliα

Table 10: Clones from LicA1 and respective mutations and bioactivity (NA- null activity, RA- reduced activity, VLA – very low activity).

Clone	Leader peptide	Core peptide	Both (core and leader)	GG	Frame Shift	STOP codon	Bioactivity
NA_1.2_A1	-	C71Y	-	-	-	-	NA
NA_1.8_A1	-	T47M	-	-	-	-	NA
NA_1.13_A1	-	T66I	-	-	-	-	NA
NA_1.15_A1	-	C75S	-	-	-	-	NA
NA_1.16_A1	-	L64P	-	-	-	-	NA
NA_1.19_A1	-	N60K	-	-	-	-	NA
NA_1.24_A1	-	C71F	-	-	-	-	NA
NA_1.27_A1	-	T47A	-	-	-	-	NA
RA_1.23_A1	-	P57L	-	-	-	-	NA
NA_1.32_A1	-	T47A	-	-	-	-	NA
NA_1.14_A1	-	S74C	-	-	-	-	NA
NA_1.34_A1	-	C75R	-	-	-	-	NA
NA_1.36_A1	-	T47R	-	-	-	-	NA
NA_1.37_A1	-	S49R	-	-	-	-	NA
NA_1.38_A1	-	C65S	-	-	-	-	NA
NA_1.46_A1	-	E70D	-	-	-	-	NA
RA_1.14_A1	-	G62R	-	-	-	-	NA
RA_1.19_A1	-	V67E	-	-	-	-	NA
RA_1.15_A1	-	L64Q	-	-	-	-	RA
RA_1.21_A1	-	P57A	-	-	-	-	RA
RA_1.26_A1	-	T45K	-	-	-	-	RA
RA_1.27_A1	-	T45A	-	-	-	-	RA
RA_1.28_A1	-	T66R	-	-	-	-	RA
RA_1.31_A1	-	P73S	-	-	-	-	RA
VLA_1.8_A1	-	A52V	-	-	-	-	RA
VLA_1.12_A1	-	A52V	-	-	-	-	RA
VLA_1.19_A1	-	S74G	-	-	-	-	RA
VLA_1.22_A1	-	A52T	-	-	-	-	RA
RA_1.2_A1	-	A52V	-	-	-	-	RA
RA_1.9_A1	-	P73S	-	-	-	-	RA
VLA_1.37_A1	-	S49N	-	-	-	-	RA
VLA_1.42_A1	-	A52T	-	-	-	-	RA
VLA_1.15_A1	-	I53N	-	-	-	-	VLA
RA_1.11_A1	-	C51Y	-	-	-	-	VLA
NA_1.7_A1	-	Y63C	-	-	-	-	VLA
NA_1.33_A1	-	Y63C	-	-	-	-	VLA
NA_1.42_A1	-	Y63C	-	-	-	-	VLA
RA_1.10_A1	-	Y63C	-	-	-	-	VLA
NA_1.28_A1	-	Y63C	-	-	-	-	VLA
NA_1.4_A1	-	-	-	G44C	-	-	NA
RA_1.17_A1	-	-	-	G43E	-	-	RA
VLA_1.38_A1	T19I;P25Q	-	-	-	-	-	RA
RA_1.3_A1	-	I46V; V67A	-	-	-	-	RA

NA_1.11_A1	-	T47M;L64Q	-	-	-	-	NA
VLA_1.28_A1	-	T47M;L64Q	-	-	-	-	NA
RA_1.13_A1	-	-	L33P;I53F	-	-	-	RA
RA_1.25_A1	-	-	S40R;P73L	-	-	-	RA
RA_1.16_A1	-	-	Q34H;L58V	-	-	-	RA
NA_1.44_A1	-	-	G44C;L64M	-	-	-	NA
VLA_1.39_A1	-	-	A26V;P73R	-	-	-	NA
NA_1.20_A1	-	-	K6R; T47A	-	-	-	NA
NA_1.5_A1	-	-	Y17C;I68T	-	-	-	NA
VLA_1.21_A1	-	-	N28S;Y63N	-	-	-	VLA
RA_1.30_A1	-	-	K31R;S49G	-	-	-	VLA
NA_1.30_A1	-	-	-	-	Delection	-	NA
NA_1.6_A1	-	-	-	-	Delection	-	NA
NA_1.10_A1	-	-	-	-	Delection	-	NA
NA_1.23_A1	-	-	-	-	Delection	-	NA
NA_1.21_A1	-	-	-	-	Insertion	-	NA
NA_1.9_A1	-	-	-	-	Delection	-	NA
NA_1.39_A1	-	-	-	-	Delection	-	NA
NA_1.40_A1	-	-	-	-	Delection	-	NA
NA_1.21_A1	-	-	-	-	Insertion	-	NA
NA_1.17_A1	-	-	-	-	-	G43STOP	NA
NA_1.31_A1	-	-	-	-	-	L54STOP	NA
NA_1.35_A1	S40R	-	-	-	-	G59STOP	NA
NA_1.43_A1	-	L48P	-	-	-	Y17STOP	NA
NA_1.1_A1	-	(I53V)	-	-	-	E32STOP	NA
RA_1.3_A1	-	I46V; V67A	-	-	-	-	NA

Appendix 6 - Clones and respective mutations and bioactivity of Bliβ

Table 11: Clones from LicA2 and respective mutations and bioactivity (NA- null activity, RA- reduced activity, VLA – very low activity).

Sample	Leader peptide	Core peptide	Hexapeptide	Both (core and leader)	GG	Frame Shift	STOP codon	Bioactivity
NA_1.6_A2	-	T55R	-	-	-	-	-	NA
NA_1.11_A2	-	T43R	-	-	-	-	-	NA
NA_1.16_A2	-	T67I	-	-	-	-	-	NA
NA_1.19_A2	-	G57D	-	-	-	-	-	NA
NA_1.21_A2	-	T67A	-	-	-	-	-	NA
NA_1.24_A2	-	C70S	-	-	-	-	-	NA
NA_1.26_A2	-	C53F	-	-	-	-	-	NA
NA_1.28_A2	-	S50P	-	-	-	-	-	NA
NA_1.29_A2	-	T71A	-	-	-	-	-	NA
RA_1.20_A2	-	T59M	-	-	-	-	-	NA
VLA_1.9_A2	-	C65R	-	-	-	-	-	NA
VLA_1.8_A2	-	C65R	-	-	-	-	-	NA
VLA_1.23_A2	-	T59P	-	-	-	-	-	NA
VLA_1.25_A2	-	T67R	-	-	-	-	-	NA
VLA_1.28_A2	-	W51C	-	-	-	-	-	NA
NA_1.2_A2	-	C65S	-	-	-	-	-	NA
NA_1.3_A2	-	C74R	-	-	-	-	-	NA
NA_1.5_A2	-	T71I	-	-	-	-	-	NA
NA_1.24_A2	-	C70S	-	-	-	-	-	NA
NA_1.27_A2	-	A62D	-	-	-	-	-	NA
NA_1.7_A2	-	C65S	-	-	-	-	-	NA
NA_1.31_A2	-	T71R	-	-	-	-	-	NA
NA_1.36_A2	-	T67I	-	-	-	-	-	NA
NA_1.41_A2	-	T67A	-	-	-	-	-	NA
NA_1.42_A2	-	C65S	-	-	-	-	-	NA
RA_1.6_A2	-	V60D	-	-	-	-	-	NA
VLA_1.7_A2	-	K69E	-	-	-	-	-	NA
VLA_1.27_A2	-	K69E	-	-	-	-	-	NA
NA_1.20_A2	-	A46P	-	-	-	-	-	VLA
VLA_1.4_A2	-	I54T	-	-	-	-	-	VLA
VLA_1.12_A2	-	G57C	-	-	-	-	-	VLA
RA_1.17_A2	-	T68I	-	-	-	-	-	VLA
VLA_1.29_A2	-	V39G;P45H	-	-	-	-	-	VLA
RA_1.15_A2	-	P45H	-	-	-	-	-	RA
RA_1.18_A2	-	T48A	-	-	-	-	-	RA
RA_1.19_A2	-	K69M	-	-	-	-	-	RA
RA_1.7_A2	-	S72R	-	-	-	-	-	RA
VLA_1.11_A2	-	P45H	-	-	-	-		RA
VLA_1.18_A2	-	T55I	-	-	-	-		RA
VLA_1.22_A2	-	T68A	-	-	-	-		RA
VLA_1.30_A2	-	T68A	-	-	-	-		RA
RA_1.21_A2	-	-	P41H	-	-	-	-	RA
NA_1.15_A2	-	-	E42K	-	-	-	-	RA

RA_1.27_A2	-	-	-	-	G36V	-	-	RA
VLA_1.26_A2	A22E	-	-	-	-	-	-	NA
RA_1.23_A2	P21T	-	-	-	-	-	-	RA
RA_1.29_A2	P21S	-	-	-	-	-	-	RA
RA_1.30_A2	H20R	-	-	-	-	-	-	RA
NA_1.23_A2	-	-	-	L30S;G57D	-	-	-	NA
VLA_1.19_A2	-	-	-	M6T; T43A	-	-	-	NA
RA_1.26_A2	-	-	-	P21Q;S61F	-	-	-	NA
VLA_1.13_A2	-	-	-	A10V;C65S	-	-	-	NA
RA_1.2_A2	-	-	-	N19K;L64I	-	-	-	RA
RA_1.12_A2	-	-	-	L33F;A56V	-	-	-	RA
RA_1.10_A2	-	-	-	P21Q;M24I	-	-	-	VLA
RA_1.11_A2	N19D	-	-	-	G35E	-	-	RA
VLA_1.17_A2	R12H	-	-	-	-	G36R	-	VLA
VLA_1.10_A2	-	L64S	-	-	N40I	-	-	VLA
NA_1.32_A2	-	-	-	-	-	Delection	-	NA
NA_1.35_A2	-	-	-	-	-	Delection	-	NA
NA_1.37_A2	-	-	-	-	-	Delectio	-	NA
NA_1.39_A2	-	-	-	-	-	Delection		NA
NA_1.30_A2	-	-	-	-	-	-	G36STOP	NA
NA_1.9_A2	-	-	-	-	-	-	R73STOP	NA
NA_1.4_A2	-	-	-	-	-	-	R73STOP	NA
NA_1.18_A2	-	-	-	-	-	-	L33STOP	NA
NA_1.33_A2	V34L	-	-	-	-	-	K31STOP	NA
NA_1.34_A2	V34A	-	-	-	-	-	L33STOP	NA
NA_1.38_A2	H20L	-	-	-	-	-	G17STOP	NA
NA_1.43_A2	-	-	-	-	-	-	R73STOP	NA
NA_1.17_A2	A18P	-	-	-	-	-	G35STOP	NA
NA_1.25_A2	-	C53F	-	-	-	-	R73STOP	NA
VLA_1.15_A2	H20Y;E27G	-	N37D	-	-	-	-	VLA